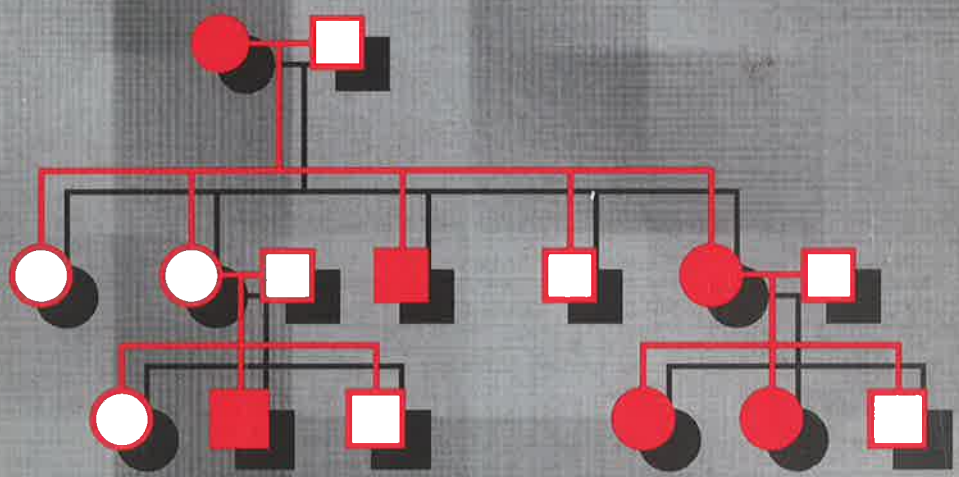
Histidine-rich glycoprotein and thrombosis



Bart Hennis

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Stellingen

Behorende bij het proefschrift "histidine-rich glycoprotein and thrombosis"

Het tot nu toe gepubliceerde onderzoek levert geen bewijs voor het bestaan van een causaal verband tussen verhoogde plasmaniveaus van histidine-rijk glycoproteine en veneuze trombose.

Uit de waarneming dat EDTA de interactie tussen histidine-rijk glycoproteine en fibrinogeen verstoort mag niet geconcludeerd worden dat calcium ionen voor de binding noodzakelijk zijn (Leung, 1986, *J. Clin. Invest.* **77,** 1305-1311 en van Boheemen et al., 1994, *Fibrinolysis* **8 suppl 1,** 94, abstract:262).

Histidine-rijk glycoproteine wordt niet in megakaryocyten of bloedplaatjes gesynthetiseerd (Leung et al., 1983, *Blood,* **62,** 1016-1021).

Omdat de expressie van ApoA-I en ApoA-IV in de darmen van ApoC-III knock-out muizen verlaagd is, kan het uitblijven van hypertriglyceridemie na de toediening van een vetbolus niet zonder meer worden toegeschreven aan de afwezigheid van ApoC-III (Maeda et al., 1994, *J. Biol. Chem.* **269,** 23610-23616).

Het feit dat de reactiesnelheid van PAI-2 met t-PA lager is dan die met u-PA wil nog niet zeggen dat PAI-2 een specifieke remmer van u-PA is (Jessup, 1994, *Gastroenterology* **107,** 1555-1559, editorial).

De beoogde 1 centimorgan genetische linkagemap mag niet het einddoel zijn van het Humane Genoom Project, aangezien het kloneren van genen op grond van linkage-disequilibrium een grotere markerdichtheid vereist (Hästbacka et al., 1994, *Cell* **107,** 1073-1087).

Omdat de premie voor ziektekostenverzekering van oudsher is berekend op het bestaan van erfelijke ziekten, moeten kinderen bij wie met moderne technieken een erfelijke ziekte wordt ontdekt, ook in de toekomst normaal kunnen worden verzekerd.

De dagvaarding van de dierenarts voor de tuchtraad wegens incompetent handelen wordt vaker voorafgegaan door gebrekkige communicatie tussen de dierenarts en de cliënt dan door daadwerkelijke incompetentie van de dierenarts.

Voor het goed tot expressie komen van het AIO-schap mag een promotor geen repressor blijken.

Een proband mag in statistische analyses niet als contrabande kunnen worden aangemerkt.

Gezien de risico's voor mens en ecosysteem zouden de media minder aandacht dienen te besteden aan het welbekende gat in de ozonlaag boven antarctica, maar meer aan de afname van de ozonlaag boven het noordelijk halfrond, en het meest aan de toename van ozonconcentraties aan het aardoppervlak.

De gedetailleerdheid van de projectomschrijving die tegenwoordig gevraagd wordt bij een subsidieaanvraag, verkleint de kans op subsidie voor vernieuwend onderzoek.

Ondanks de drang naar hoge arbeidsproductiviteit staat het arbeidsethos in Nederland het doen van een hazeslaapje niet toe.

Het milieu leeft niet meer.

Histidine-rich glycoprotein and thrombosis

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 2 februari 1995
te klokke 14.15 uur

door
Bart Cornelis Hennis

geboren te Utrecht in 1964

Promotiecommissie

Promotoren:	Prof. dr. P. Brakman
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Referent:	Prof. dr. R.M. Bertina
Lid:	Prof. dr. J.P. Vandenbroucke

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Contents

Chapter 1

General introduction

1.1 Introduction

Bleeding or pathological thrombus formation can be caused by an imbalance between coagulation and fibrinolysis. The process of coagulation prevents the leaking of blood from the circulation after damage of the vessel wall by formation of a fibrin clot which seals the damaged vessel wall. The fibrinolytic system dissolves the fibrin clot while the vessel wall is being repaired. Decreased activity of the coagulation system or an increase in fibrinolytic activity may result in a bleeding tendency whereas enhanced activity of the coagulation system or impaired fibrinolytic activity may lead to thrombosis. Both the fibrinolytic system and the coagulation system involve a cascade of a large number of blood and tissue-bound factors. Aberrant function or deviant plasma concentrations of any of these factors may result in impaired coagulation or fibrinolysis and thereby in a disruption of the haemostatic balance. Inherited deficiencies of protein C, protein S, antithrombin III, congenital dysfibrinogenemia (Malm et al., 1992) and recently resistance to activated protein C (Dahlbäck et al., 1993; Bertina et al., 1994) are well known disorders of the coagulation system which are associated with thrombosis. On the other hand, impaired fibrinolytic activity has been associated with postoperative deep venous thrombosis (reviewed in Prins et al., 1991). In addition patients with thrombosis have often higher plasminogen activator inhibitor 1 (PAI-1) levels than controls (Malm et al., 1992). However, although in several studies congenital deficiencies of plasminogen (Dolan et al., 1988; Shigekyo et al., 1992), tissue-type plasminogen activator (t-PA) or inherited elevated PAI-1 levels (reviewed in Prins et al., 1991) have been reported no clear evidence is found for the association of inherited deficiencies of fibrinolytic factors and thrombosis.

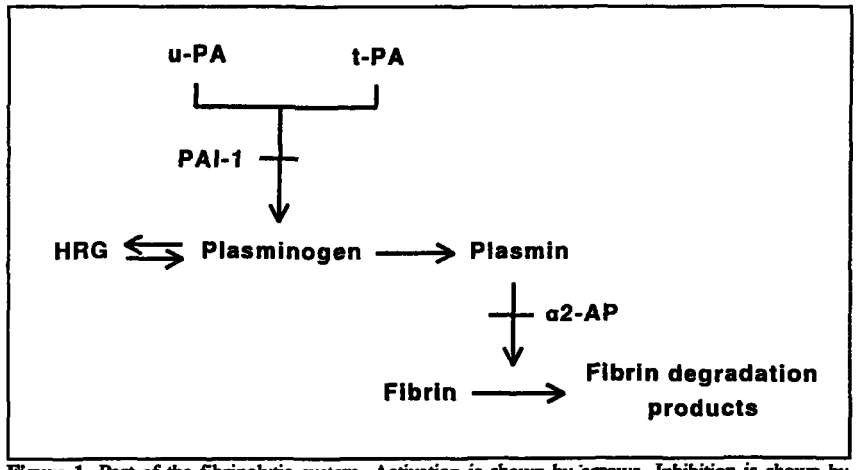

Figure 1. Part of the fibrinolytic system. Activation is shown by arrows. Inhibition is shown by bars crossing arrows. A double arrow indicates the formation of a reversible complex. u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1; HRG, histidine-rich glycoprotein; a2-AP, a2-antiplasmin.

In theory impaired fibrinolysis can be caused by plasminogen deficiency, decreased t-PA or urokinase type plasminogen activator (u-PA) levels and increased a_2-antiplasmin or PAI-1 levels which all result in decreased plasmin activity. In the last 10 years, histidine-rich glycoprotein (HRG) has come forward as a new factor elevated levels of which are possibly associated with lower fibrinolytic activity. The reversible complex formation of HRG with plasminogen may interfere with an efficient activation of plasminogen and therefore cause a decrease in fibrinolytic activity (figure 1). In the following paragraphs the identity and several physiological interactions of HRG will be presented.

1.2 HRG gene and protein structure

Gene

Up to now only the cDNA sequence of HRG has been published (Koide et al., 1986). The messenger encoding HRG comprises 2067 base pairs and although three different polyadenylation sites can be recognised in the sequenced CDNA clones, only one MRNA transcript is observed (Hennis et al., 1991). The structural gene encoding HRG has been characterized partially. It is about 10 kb in length and contains seven exons and six introns (Koide, 1988; Wakabayashi and Koide, unpublished results). The gene is localized on the long arm of chromosome 3 at 3q21-qter (Van den Berg et al., 1990).

Protein

Initially HRG was isolated as a 3.8 S-a_2-globulin with a molecular weight of 58,000 Da (Haupt et al., 1972; Heimburger et al., 1972). During this first purification of HRG a partially degraded protein had been obtained. The isolation of the first intact single chain HRG was reported by Rylatt et al. (1981). Since then the molecular weight of native HRG on SDS-PAGE is considered to be 75,000 Da under reducing conditions (Koide et al., 1985; Yip et al., 1991) whereas under non-reducing conditions its molecular weight is about 72,000 Da (Koide et al., 1985). The exact molecular weight is 66,100 Da which has been determined by matrix-assisted UV laser desorption time-of-flight mass spectrometry (Yip et al., 1991).
Several physical and chemical properties of HRG have been determined. Unfortunately, this was done using partially degraded HRG (Heimburger et al., 1972). This HRG consists of several fractions with isoelectric points between 5.6 and 6.2 and has a carbohydrate content of about 14%. The extinction coefficient $E1\%_{280\ nm,\ 1\ cm}$ is 5.85. As yet it is not clear whether these properties can be applied to native HRG although a total carbohydrate content of 13% can be calculated from the molecular weight of the amino acid chain (57,646 Da) (Koide et al., 1986) and the molecular weight of the native protein (66,100 Da) (Yip et al., 1991).

Primary structure

After the cDNA for HRG was cloned by Koide et al., (1986) the amino acid composition could be fully derived. The 2067 bp cDNA which was obtained from a human liver cDNA library encodes a protein of 507 amino acids preceded by a signal peptide of 18 amino acids. HRG takes its name from the remarkably high content of histidines (13%) in the protein, although it contains also 13% of prolines.

Another striking feature is the organisation of HRG in multiple domains (Koide et al., 1986). In figure 2 two cystatin domains, two proline-rich domains and a histidine-rich domain are shown. The histidine-rich domain (residues 330-389) is made up of a repetition of 12 blocks of 5 amino acids. The consensus sequence Gly-His-His-Pro-His is highly conserved at the amino acid level (80%) and even more at the nucleotide level (88%).

Four sites with the consensus sequence Asn-X-Ser/Thr for N-linked carbohydrates are present in the molecule (Koide et al., 1986) but the localization and nature of the bound carbohydrates is unknown.

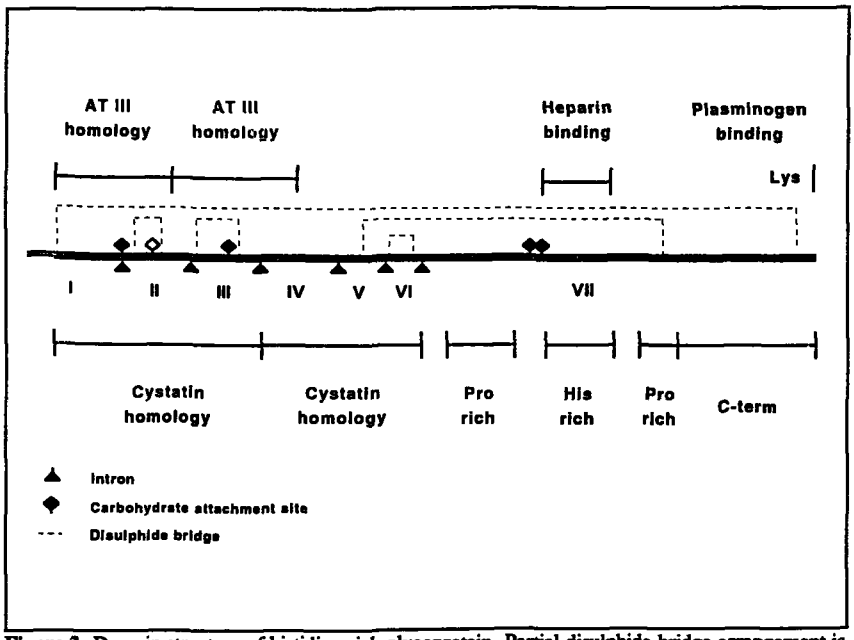

Figure 2. Domain structure of histidine-rich glycoprotein. Partial disulphide bridge arrangement is based on homology with bovine HRG. Roman numerals indicate exons. AT III, antithrombine III; Pro-rich, proline-rich region; his-rich, histidine-rich region; C-term, C-terminal region; Lys, C-terminal lysine.

Secondary structure
HRG turns out to be hydrophilic throughout the entire molecule (Koide et al., 1986). Especially the histidine-rich region is highly hydrophilic and will most probably be surface exposed (Yip et al., 1991). Less than 15% helical structure, 32% b-sheets, 22% b-turns and 31% other structures and random coils are present in HRG (Koide et al., 1986; Morgan et al., 1978; Yip et al., 1991).

Tertiary structure
As yet the disulphide bridge arrangement of human HRG has not been determined. However, it has been suggested that five of the disulphide bridges which are present in bovine HRG are also present in human HRG (Sørensen et al., 1993). One bridge connects the N-terminal part to the C-terminal part (confirmed for human HRG by van Boheemen, unpublished results), two are located in the first cystatin domain, one in the second cystatin domain and another bridge connects the second cystatin domain with the second proline-rich domain (figure 2).

Homology to cysteine protease inhibitors
Although HRG has no known protease inhibitor activity (Turk et al., 1991) HRG is thought to be evolutionarily related to members of the cystatin superfamily of cysteine proteinase inhibitors (Rawlings et al., 1990). HRG contains two tandemly-repeated cystatin-like domains of 112 amino acids that have about 10%-25% amino acid homology to the cystatin domains of several members of the cystatin superfamily (Koide et al., 1987) (figure 2).

HRG is especially homologous to a_2-HS-glycoprotein (AHSG) and high-molecular weight kininogen (HMWK) which are also members of the cystatin superfamily (Rawlings et al., 1990). Similar to HRG, AHSG has two cystatin domains which are not active as proteinase inhibitor whereas HMWK has only one domain (the first of three) that is not active. Apart from the homology between the cystatin domains of HMWK and HRG, homology is found between the histidine-rich regions of the two proteins. In this region about 50% of the amino acids are identical (Koide et al., 1986). The disulphide loop arrangement in the cystatin domains of AHSG, HMWK and HRG is also homologous (Sørensen et al., 1993; Koide et al., 1987). Markedly, the first cysteine residue at the N-terminus is connected to a cysteine residue near the C-terminus in all three proteins.

The localization of introns in the cystatin domains of the HRG gene is essentially identical to HMWK and other members of the cystatin superfamily (Koide, 1988). Furthermore it is interesting to note that the genes of 4 members of the cystatin superfamily are located on the distal section of the long arm of chromosome 3. Stefin A (STF1) is localized on 3q21 (Naylor et al., 1991), Kininogen (KNG) on 3q26-qter

(Cheung et al., 1992), a_2-HS-glycoprotein on 3q27-q28 (AHSG) (Magnuson et al., 1988) and HRG on 3q21-qter (Van den Berg et al., 1990; Naylor et al., 1991).

1.3 Synthesis

Plasma appears to contain the most abundant pool of HRG, but HRG is also found in platelets and megakaryocytes, infant urine, colostrum and milk. The concentration of HRG in plasma is 90-125 mg/ml which is about 0.15% of total plasma proteins (Heimburger et al., 1972; Morgan et al., 1978; Lijnen et al., 1981; Yip et al., 1991). HRG is stored in the a-granules of platelets at a concentration of 0.4 mg/10^9 platelets which is in blood 0.1% of the plasma HRG concentration (Leung et al., 1983; Hoffmann et al., 1993). Human colostrum at day 1 to 3 after parturition and human milk at day 4 to 120 contain 0.1-10 mg HRG/ml (Hutchens et al., 1992). The concentration of immuno reactive HRG in human milk-fed infant urine has not yet been determined (Yip et al., 1991).

Source of plasma HRG
In recent years several sites of synthesis of HRG have been reported. HRG is synthesized "de novo" in cultured rabbit hepatocytes (Smith et al., 1988) and HRG mRNA can be detected in cultured rat, monkey and human hepatocytes (Hennis et al., 1991). A detailed survey of the production of HRG MRNA in rat tissues indicated that the parenchymal cells of the liver are the source of plasma HRG (Hennis et al., 1991). There is no indication that plasma HRG is produced in other cells or tissues than the liver. The previously reported synthesis of HRG by murine peritoneal macrophages (Sia et al., 1982) has never been confirmed and no HRG MRNA or protein can be detected in freshly isolated human monocytes or cultured human macrophages (Hennis et al., 1991).

HRG from platelets
The molecular weight of HRG isolated from plasma or from platelets appears to be identical indicating that platelet HRG is most probably equal to plasma HRG. The a-granules of platelets contain 0.4 mg HRG/10^9 platelets which is 0.045% of all platelet proteins (Leung et al., 1983). HRG is also present in the cytosol of human megakaryocytes (Leung et al., 1983) but the origin of platelet HRG is still unknown. However, taking the ratio of the concentration of HRG in platelets and plasma into account, it is not expected that HRG is synthesized in megakaryocytes or platelets. Proteins which are produced in these cells do have very high ratios (e.g. Von Willebrand factor, thrombospondin and platelet factor 4) (George, 1990). HRG has a ratio similar to high-molecular weight kininogen of which the take-up is receptor-mediated. HRG is therefore also thought to be taken up from the plasma. This may be either by receptor-mediated or by fluid phase endocytosis.

Other sources
Intact HRG and also even smaller immunoreactive fragments can be isolated from human colostrum and milk (Hutchens et al., 1992). The origin of this substantial amount of HRG has not yet been determined. It may be synthesised in the mammary gland or it may be taken up from plasma. The source of HRG in the urine of preterm infants who are fed with human milk is unclear but may be of maternal origin (Hutchens et al., 1992).

1.4 Regulation of plasma levels

Little information is available about the regulation of plasma HRG levels. Like many other plasma proteins, HRG is synthesized by the liver but it is unclear whether the clearance of HRG also takes place in the liver.

Plasma HRG levels
Plasma HRG levels are normally expressed as a percentage of a pool of plasmas obtained from healthy individuals. To define a normal range for HRG levels, distributions of plasma HRG levels have been determined in healthy volunteers. The distribution of HRG levels resembles a normal distribution but is slightly skewed to the right indicating a higher prevalence of high HRG levels (Lijnen et al., 1981; Samama et al., 1983; Engesser et al., 1987; Hoffmann et al., 1993; Ehrenforth et al., 1994). However, no indication of a bimodal distribution is found in these studies. The reported normal ranges are very similar and by taking all studies together a mean normal range of 55%-149% can be calculated. Serum HRG levels are comparable with plasma levels indicating no significant loss of HRG during coagulation (Saigo et al., 1990).
Individual HRG levels appear to fluctuate little with time. The intra-individual variance is only 1% of the total variance whereas the inter-individual variance accounts for 97% of the total measured variance (de Bart et al., 1992).
Low HRG levels are found in young children and a strong correlation between HRG levels in foetuses and the time of gestation is found. Before 35 weeks of gestation foetal HRG levels are not detectable whereas at the time of birth the HRG level is 10%-20% of the adult level (Corrigan et al., 1990; Caccamo et al., 1992). A gradual increase of the level is observed leading to a HRG level of about 60% at the age of 3. (Corrigan et al., 1990). Children aged 10-15 years have on average 20% lower levels than adults (Castaman et al., 1993). No age effects have been reported in groups of healthy adults (Morgan et al., 1978; Hoffmann et al., 1993).

Clearance
The turnover of HRG has been determined by using radioactive labelled HRG which was partially degraded (Lijnen et al., 1981). The turnover of this HRG is about 3 days which is similar to the turnover of some other hepatic plasma proteins like antithrombine III,

plasminogen and a_2-antiplasmin. It is not known whether the turnover of intact HRG is different.
The turnover of HRG during streptokinase infusion increases with 100% from a half-life of 3 to 1.5 days (Lijnen et al., 1981). However, no differences in HRG levels were found before and during the therapy (Lijnen et al 1980; Hoffmann et al., 1990; Goodnough et al., 1985). Unfortunately, partially degraded HRG has also been used to determine the turnover of HRG during thrombolytic therapy. But when intact HRG has a similar susceptibility to proteolytic degradation during streptokinase therapy as the partially degraded HRG, then the comparable HRG levels before and during therapy indicate an increased synthetic rate of HRG.
The half life of HRG in heparinized DVT patients is also decreased from 3 days to 2 days (Lijnen et al., 1983). The increased turnover may be due to a faster clearance of the heparin-HRG complex as is also seen for AT III during heparin therapy. HRG plasma levels do not change during heparin therapy indicating an increased synthesis. However, as the turnover during heparin therapy is also determined using a partially degraded HRG it is possible that only complexes of heparin and degraded HRG are cleared faster, leaving the level of native HRG intact.

Effect of hormones
HRG levels are susceptible to both exogenous and endogenous hormones. Sex hormones especially are involved in the regulation of plasma HRG levels. Women using oral contraceptives show a decrease of 15%-30% in their HRG level (Jespersen et al., 1982; Gevers Leuven et al., 1987; Jespersen et al., 1990). Since no effect has been found for progestogens (Haukkamaa et al., 1983), this effect may be ascribed to the estrogen component. A comparable effect is observed during pregnancy when there is an increased oestrogen secretion. A gradual almost linear decline in HRG levels starts at the beginning of the second trimester of pregnancy and progresses to about 50% of the mean adult HRG concentration at parturition. Levels turn to normal within two weeks after delivery (Morgan et al., 1978; Haukkamaa et al 1983; Omri et al., 1988; Castaman et al., 1993). The effect caused by reduced oestrogen secretion is observed in women after the menopause. Postmenopausal women do have a significantly higher HRG level than premenopausal women and men in the same age category (Thompson et al., 1991). Although the effect caused by endogenous sex hormones is quite obvious, male HRG levels are not different from female HRG levels before the menopause (Morgan et al., 1978; Hoffmann et al., 1993). The anabolic agent stanozolol (Kluft et al., 1984) and the immuno suppressive agent prednisone (Morgan et al., 1986) both reduce HRG levels by 20%-30%, indicating that hormones other than estrogen also produce an effect on the HRG level.

Negative acute-phase response
Both on the protein level and on the mRNA level HRG appears to be a negative acute-phase reactant. Turpentine treatment of rabbits lowers the liver mRNA for HRG 4-5 fold

(Smith et al., 1988) and HRG is negatively correlated with serum C-reactive protein (CRP) (r=-0.45) which is a strong positive acute-phase reactant (Saigo et al., 1990). Another member of the cystatin superfamily, AHSG is also produced by the liver and gives also a negative acute-phase response (Bradley et al., 1977) possibly indicating a similar regulatory mechanism.

1.5 Interactions and functions

In the last two decades several functions have been assigned to HRG. Prothrombotic properties due to the binding of HRG to plasminogen and heparin have been proposed whereas the binding of HRG to fibrinogen and fibrin may have an effect on fibrin polymerization. The strong binding capacity of zinc and other divalent metal ions, suggests a role in metal metabolism as a metal transport and storage protein. On the other hand, the inhibitory activity of HRG on T cell activation and proliferation indicates a role in the immune system. Although the function of HRG is unclear the multiple domain structure may indicate a role of HRG in diverse physiological systems.

Heparin binding

Initially the affinity of HRG for heparin was thought to be due to the homology with antithrombin III (AT III). Amino acids 1-64 and 65-146 of HRG have about 40% homology with amino acids 24-79 and 80-139 of AT III, respectively (Koide et al., 1986) (see also figure 2). However, the interaction of heparin with HRG appears to be different from the interaction with AT III (Lijnen et al., 1983). Both heparin with a low affinity for AT III and heparin with a high affinity for AT III bind HRG with equal affinity, but in contrast to AT III, the interaction of HRG and heparin can be inhibited by EDTA. This suggests that divalent cations are involved in the interaction and that the histidine-rich domain, which binds divalent cations (see also below: binding of divalent metal ions), may also play a role in this interaction (reviewed in Lijnen et al., 1989). More evidence for the involvement of the histidine-rich domain in the interaction with heparin is provided by experiments with rabbit HRG: modification of all histidine residues eliminates the heparin binding (Burch et al., 1987). Although the presence of an AT III like heparin binding site is not excluded, the hydrogen bonding between protonated histidine residues and negatively charged groups as sulphate and carboxyl are likely to be the cause of the interaction between HRG and heparin.

In purified systems HRG and high affinity heparin react with apparent 1:1 stoichiometry to form a complex with a dissociation constant of 7 nM, compared to a dissociation constant of 65-200 nM for the complex between AT III and heparin (Lijnen et al., 1983). This means that at least *in vitro* HRG may be a good competitor for the binding to heparin.

The possible role of HRG in neutralizing the anticoagulant activity of heparin in plasma by competition with AT III, has been investigated by incubation of total plasma to immobilized unfractionated heparin. HRG appears, after AT III, to be the most abundant plasma protein binding to heparin (Dawes, 1993). Similar results are found for low molecular weight heparin (LMWH) although the affinity of HRG for LMWH is lower (Dawes et al., 1993; Lane et al., 1986). HRG is therefore the only protein likely to neutralize a significant proportion of the anticoagulant activity of heparin by direct competition with AT III. However, HRG levels are not different in heparin resistant patients (Young et al., 1992) and the variation in HRG level accounts only for a small amount of the variation in anticoagulant activity of heparin in patients (Prins et al., 1993). The latter results indicate only a minor role for HRG in the physiological neutralization of the anticoagulant activity of heparin in spite of the high affinity for heparin observed *in vitro*. The differences of anticoagulant activity of heparin in patients may therefore be caused by factors other than HRG.

Plasminogen activation

The first indication that HRG may play a role in the fibrinolytic system was found by Lijnen et al., (1980). They simultaneously purified a_2-antiplasmin and HRG during chromatography on an insolubilized fragment of plasminogen (N-terminus and kringle 1-3) which contained the high affinity lysine-binding site (LBS I). The plasminogen binding site of HRG consists of its C-terminal lysine together with a yet undefined region on the N-terminal domain (Borza et al., 1994; Hennis, unpublished observations) (figure 2). In plasma, complexes of HRG and plasminogen are indeed observed after immunoprecipitation of HRG (Chang et al., 1992a).

Physiological concentrations of HRG have been found to reduce the amount of plasminogen binding to fibrin by 50% in fibrin clotting experiments in the presence of plasminogen. From these experiments, an apparent K_d of about 1mM can be calculated for the reversible complex of plasminogen and HRG (Lijnen et al., 1983). A similar K_d (0.5 mM) is found in affinity crossed immunoelectrophoresis experiments for both Glu and Lys plasminogen (Kluft et al., 1988). On the other hand, a much smaller decrease in the plasminogen binding to fibrin of 13% has been reported by Ichinose et al., (1984) in a similar system as used by Lijnen et al (1983). Furthermore, in a different system mimicking the fibrin/plasma interface, HRG also hardly decreases the amount of plasminogen bound to fibrin (Anglés-Cano et al., 1992). All experiments mentioned so far were performed in purified systems and may therefore not represent the actual situation in the circulation. Nevertheless, assuming a K_d of 1 mM initially found for HRG by Lijnen et al. (1980) and a K_d of 4 mM for a_2-antiplasmin it can be calculated that 50% of plasma plasminogen may be in complex with HRG.

The affinity of HRG for the lysine-binding sites of plasminogen and plasmin has also consequences for the interaction between a_2-antiplasmin and plasmin and for the activation of plasminogen. HRG reduces the apparent rate constant of the reversible complex formation between plasmin and a_2-antiplasmin by competition for the high

affinity lysine-binding site (Lijnen et al., 1980). The apparent K_d of the interaction between HRG and plasmin is about 0.8 mM which is very close to the apparent K_d found for the HRG-plasminogen complex (Lijnen et al., 1983; Ichinose et al., 1984). HRG reduces the activation of plasminogen by tissue-type plasminogen activator (t-PA) as measured by the plasmin activity using fibrin as a substrate (Lijnen et al., 1980). One explanation of this reduced plasmin activity may be that the interaction of HRG and plasminogen prevents binding of plasminogen to fibrin. The apparent K_d of the single association reaction between HRG and plasminogen in these experiments is about 1.1 mM. Another mechanism which explains the reduction in fibrinolytic activity due to HRG is proposed by Liu et al., (1992). They report that HRG accelerates the activation of plasminogen by 300% when the chromogenic substrate S-2251 is used, but that HRG decreases the activity of plasmin with 20%-30% when fibrin is the substrate. Both Lijnen et al., (1980) and Liu et al., (1992) find no effect of HRG on plasmin activity when S-2251 is the substrate. It is suggested that t-PA activity towards plasminogen is increased by conformational changes induced by HRG binding, but that this binding on the other hand inhibits plasmin activity towards fibrin. More evidence for the hypothesis that HRG does not inhibit fibrinolysis by hampering the binding of plasminogen to fibrin is provided by Anglés-Cano et al. (1992 and 1993). In their system HRG hardly decreases the binding of plasminogen to a thrombin treated fibrinogen monolayer. In addition, HRG does not decrease the subsequent plasmin activity towards the chromogenic substrate CBS 1065. However, they also found no decrease in generation of carboxy-terminal lysines after activation of plasminogen in the presence of HRG using the thrombin treated fibrinogen monolayer as the substrate, whereas in the presence of a_2-antiplasmin an important limitation of carboxy-terminal lysines is observed. This may indicate that in this system HRG does not inhibit plasmin activity towards fibrin. In conclusion, there is ambiguous evidence for the inhibition of fibrin degradation by HRG. So far, it seems that the observed inhibition is probably not due to a decreased binding of plasminogen to fibrin but may be explained by inhibition of plasmin activity.
Another way to enhance plasminogen activation involving HRG is the formation of a trimolecular complex of HRG, plasminogen and thrombospondin. This complex gives a more than 30 fold increase of t-PA dependent plasminogen activation compared to fluid phase activation. Although the physiological function of this complex formation is still unknown, plasminogen immobilized on thrombospondin and HRG could generate plasmin in a fibrin-free environment in a kinetically favourable manner (Leung et al., 1984; Silverstein et al., 1985 and 1985).

Fibrinogen/fibrin binding
Reversible complex formation between HRG and adsorbed fibrinogen has been demonstrated using an enzyme-linked immunosorbent assay (Leung, 1986). The interaction has an apparent K_d of about 7 nM, is divalent cation dependent and ionic in nature indicating possible involvement of the histidine-rich domain. The involvement of divalent cations has recently been confirmed by van Boheemen et al., (1994) who report

that binding of HRG with fibrinogen and fibrin is dependent on Zn(II) and not on Ca(II). The interaction between HRG and fibrinogen has also been studied using analytical ultracentrifugation (Saez et al., 1994). In this system HRG and fibrinogen form a reversible complex with a K_d of 0.40 mM and a stoichiometry of 1:1. The interaction is independent of carbohydrate side chains on the HRG molecule and is not influenced by hexanoic acid. The latter observation indicates that different binding sites exist for HRG and plasminogen on the fibrinogen surface. Indeed a trimolecular complex of plasminogen, HRG and fibrinogen can be formed (Saez et al., 1994).
The HRG-fibrinogen interaction has an effect both on the kinetics of fibrin formation and the structure of the fibrin clot as demonstrated by a prolongation of the thrombin time and a decreased final absorbance of fibrin gels, respectively (Leung, 1986). HRG is incorporated into fibrin clots in a concentration-dependent and saturable manner, with an apparent K_d of 0.25 mM. Since the extent of fibrin polymerization is not influenced by HRG it is suggested that the fibrin formed is distributed over more, but thinner fibrils in the presence of HRG. A decrease in HRG level of 20% after clotting was observed (Leung et al., 1986). However, it should be mentioned that the clotting experiments were not done by making normal serum. Leung et al. used recalcified EDTA plasma and added thrombin to obtain serum. Taking the Zn(II) dependent binding of HRG to fibrinogen and fibrin into account, it is possible that EDTA and/or metal ions have an effect on the amount of HRG which is incorporated into the fibrin clot.

Binding of divalent metal ions
HRG has been shown to bind up to 12 g-atoms of divalent metal cations per mole of protein with an affinity of K_d = 0.2-10 mM. Cu(II) and Zn(II) are the strongest binders (Morgan, 1981) and in fact, the competition between HRG and other serum proteins for Zn(II) is readily apparent *in vitro* (Guthans et al., 1982). HRG appears to be the plasma protein with the highest affinity for immobilized Zn(II) ions (Yip et al., 1991).
It is hypothesized that the histidine-rich region is responsible for binding divalent metal ions. The histidine and proline residues in the C-terminal half of the molecule are arranged in such a way that many of the histidine residues may be exposed to the aqueous environment (Loomis et al., 1988). Indeed, synthetic peptides mimicking the Gly-His-His-Pro-His (GHHPH) repeat from the histidine-rich region of HRG (Koide et al., 1986) bind 1 Cu(II) per repeat unit (Hutchens et al., 1991) and also bind Zn(II) (Hutchens et al., 1992). In addition Zn(II) binding is decreased after chemical modification of histidine residues (Morgan, 1981).

With respect to the metal binding capacity of HRG some investigators believe that HRG has a role as a metal transport or storage protein. The presence of HRG in human colostrum and milk may point towards a role for HRG among the proteins that participate in the bioavailability of divalent metal cations (Hutchens et al., 1992).

Immune system

In 1981 a human plasma factor was isolated which can inhibit the ability of lymphocytes to bind autologous erythrocytes, a phenomenon termed autorosetting (Rylatt et al., 1981). Later this autorosette inhibition factor (AIF) was identified as HRG (Lijnen et al., 1983). Intact HRG, but not proteolytically degraded HRG, gives 100% inhibition of autorosette formation even down to 0.05 mM (Lijnen et al., 1983).

Recently, the binding of HRG to human T lymphocytes has been reported. HRG binds to a 56 kDa protein on the surface of T cells which is not related to CD2. The binding is not influenced by fibrinogen and heparin suggesting that the T cell-binding and the fibrinogen- and heparin-binding sites are located in different parts of the HRG molecule (Saigo et al., 1989). The interaction of HRG with T cells suppresses proliferation of antigen receptor (CD3)-triggered T cells induced by interleukin 2, and inhibits interleukin 2 receptor expression on activated T cells, which causes a decreased T cell interferon-g release and an altered T cell dependent inhibition of erythropoiesis (Shatsky et al., 1989). The inhibition of T cell activation and proliferation is probably due to interference of HRG with protein kinase C activity (Lamb-Wharton et al., 1994).

Furthermore, recent studies report the regulation of mouse macrophage Fc receptor expression and phagocytosis by HRG (Chang et al., 1992), and the regulation of complement function by HRG via interactions with C8, C9, factor D and S-protein of the complement pathways (Chang et al., 1992). In spite of the many possible interactions that have been described, the physiological relevance of the postulated role of HRG in the immune system remains unclear.

Platelet HRG

Platelets may be an important source of HRG in the microenvironment of the haemostatic plug. Eighty percent of the stored HRG is released from the a-granules upon thrombin stimulation which may lead to a platelet HRG concentration up to 0.94 mM in the haemostatic plug (Leung et al., 1983) compared to a plasma concentration of 1.8 mM.

Due to the interaction of HRG with fibrin and plasminogen, this release of HRG during haemostatic plug formation may have important consequences for fibrin formation and for subsequent fibrinolytic processes. Furthermore, it is interesting to mention that HRG binds to stimulated platelets (Lerch et al., 1988). In the presence of Ca(II) and Mg(II) up to 21 mg of HRG is bound per 10^9 platelets without reaching saturation. The binding of HRG to platelets and the interaction between HRG and fibrin may also point towards a role of HRG in the cell-cell and cell-matrix interactions of platelets.

1.6 HRG levels during disease

The proposed functions of HRG mentioned above may be related to physiological systems in which altered HRG levels are causally related to illness although the altered

levels may also be a side effect of a disease. To further investigate the role of HRG, measurement of plasma HRG levels in patients with different diseases may give important indications of the function of HRG. In healthy volunteers HRG levels are found to be rather constant but in patients both lowered and elevated HRG levels are found.

Liver disease and sepsis

Since HRG is produced solely by the liver it is expected that HRG levels are deviant during liver malfunction. Remarkably, patients with a mild form of liver cirrhosis (Child A) have an elevation of their HRG level of about 25%. In contrast, a strong decrease in HRG levels of 50% is found both in patients with moderate (Child B) and severe (Child C) liver cirrhosis (Leebeek et al., 1989). The increase of the HRG level in Child A cirrhosis cannot be the result of an acute phase reaction, because HRG is known as an negative acute phase reactant (Smith et al., 1988; Saigo et al., 1990). Theoretically, an influence by hormones on the remaining functioning hepatocytes may be responsible for the increase. The changes of HRG levels during Child B and C liver cirrhosis may be explained by a reduced synthesis capacity of the liver, although no linear decline comparable to albumin is observed (Leebeek et al., 1989).

A marked lowering of HRG levels to 30%-40% of the normal level is also observed during septic shock (Karges et al., 1986; Lijnen et al., 1981). In one patient a striking decline from 130% to about 30% was observed within the first 7 hours of the septic shock (Karges et al., 1986). Whether this is due to an accelerated clearance or proteolytic degradation of HRG is unclear.

Coronary disease

HRG levels in patients with unstable angina pectoris or an acute myocardial infarction are not different from controls. Also, in patients with a mycardial infarction no different HRG levels are observed before and several days after the event. However, during the acute stage of the myocardial infarction HRG levels are decreased, which may be due to a negative response on the acute-phase (Six, 1989; Gram et al., 1983). Patients who develop deep venous thrombosis during the acute stage of the infarction have higher HRG levels than patients without this complication (Gram et al., 1983). No difference in HRG levels is found between patients with and without reocclusion (Gram et al., 1987; Hoffmann et al., 1991).

During thrombolytic therapy with streptokinase HRG is rapidly cleaved and almost no intact HRG is left after 30 minutes. The pattern of the degradation products is very similar to that generated by digestion of HRG with plasmin indicating that the cleavage is caused by plasmin activated by plasminogen-streptokinase complexes (Smith et al., 1985) (see also section: clearance).

Venous thrombosis

A higher prevalence of elevated HRG levels is found in patients with venous thrombosis. In three studies 5.9% (Engesser et al., 1987), 8.7% (Samama et al., 1983) and 10.8% (Ehrenforth et al., 1994) of the patients had HRG levels above the normal range. The normal range was defined by taking the extremes from the control group (Engesser et al., 1987) or by taking the mean $\pm$ 2*SD (Samama et al., 1983; Ehrenforth et al., 1994). The data from these cross-sectional studies may indicate a relationship between elevated levels of HRG and thrombosis, but whether this is a causal or coincidental relationship cannot be concluded from these studies.

Up to now, six families with an inherited elevation of HRG levels in combination with a family history of thrombosis have been reported (Falkon et al., 1991; Anglés-Cano et al., 1993; Castaman et al., 1993 (two families); Engesser et al., 1987; Ehrenforth et al., 1994). Familial venous thrombosis was diagnosed in five families whereas in one family a familial predisposition for myocardial infarction was reported (Ehrenforth et al., 1994). In five of the six families no other risk factors for thromboembolic disease were found at the time of investigation. In one family both elevated levels of HRG and plasminogen activator inhibitor 1 (PAI-1) were found in several family members with thrombosis (Anglés-Cano et al., 1993). Since increased PAI-1 levels have been associated with thrombosis (Prins et al., 1991), the elevated HRG levels may reinforce the effect of elevated PAI-1 levels and the combined effects may contribute to the venous thromboembolic events in this family.

In all six families described so far both the elevated HRG levels and thrombosis seem to be inherited, indicating that genetic factors play a role in both phenomena. Remarkably, all family members who are affected do have elevated levels of HRG, but there are also quite a few family members with elevated HRG levels who are healthy. This means that in the families reported there is only a partial association between elevated HRG levels and thrombosis. However, on the basis of the family data presented it is not possible to draw conclusions about a possible causal relationship between the two phenomena.

1.7 Scope of this thesis

Since the first purification of HRG in 1972 a variety of biological interactions and physiological functions of HRG have been described. At the moment it is still unclear what the exact function or the most important interaction of HRG is. For several years now different investigators have suggested a role for HRG in haemostasis and especially in the pathophysiology of thrombosis. Results from *in vitro* studies indicate indeed that elevated levels of HRG may decrease the fibrinolytic activity and thereby contribute to the development of thrombosis. It is postulated that the higher percentage of elevated HRG levels found in patient groups with venous thrombosis reflects this contribution. In addition several cases of inherited elevation of HRG levels have been found in families with familial thrombophilia, suggesting an association between both inherited traits.

However, in these families only a partial association was observed between elevated HRG levels and thrombosis. On basis of the available observations in case control and family studies it was not possible to discriminate whether the apparent relationship is causal or coincidental.
The aim of the present research is to further clarify the possible role of elevated HRG levels in thrombosis, particularly by adding a genetic approach. We studied the relationship between HRG and thrombosis by investigating the genetic background of HRG levels in the population and in selected families.
A summary of all data which have been reported on HRG is presented in the first chapter of this thesis. In chapter 2 the genetic and environmental influences on plasma HRG levels are investigated. The overall heritability of HRG plasma levels in the general population is studied in mono- and dizygotic twins and their parents (chapter 2.1). In this study it is found that 69% of the variance in HRG levels can be ascribed to genetic factors and 31% to environmental influences. In chapter 2.2 the effect of sex hormones, which is thought to be one of the most important environmental influences, is studied. It is found that estrogens decrease HRG levels in a dose dependent way whereas progestogens most probably do not have an effect on the HRG level. Basic research on the HRG gene to obtain suitable markers for mapping and linkage studies is reported in chapter 3. Two useful markers are identified, a Kpn I restriction fragment length polymorphism and a polymorphic dinucleotide repeat (chapter 3.1). The latter is highly informative due to the presence of multiple alleles and is used to incorporate the HRG gene in the genetic linkage map of chromosome 3 (chapter 3.2). In this chapter the physical localization of HRG is also determined using *in situ* hybridization. In subsequent studies, other polymorphisms were detected in the HRG gene of which one also has a consequence for the protein structure (chapter 4.2).

In chapter 4 molecular variants and polymorphisms of the HRG protein are described. Chapter 4.1 reports a family with inherited elevated levels of HRG which are associated with an abnormal heparin binding. Besides this possibly rare variant, a more common molecular variant which is due to an amino acid substitution, has also been identified (chapter 4.2). The relationship between plasma HRG levels and different alleles of the HRG gene is studied in chapter 5. The dinucleotide repeat is used to perform quantitative linkage analysis in a thrombophilic family with elevated HRG levels (chapter 5.1) In this family, linkage is found between a specific allele of the dinucleotide repeat and high HRG levels. In chapter 5.2 the amino acid polymorphism (chapter 4.2) is used to determine the contribution of the two molecular forms of HRG to the HRG plasma level. It is found that the two forms of HRG explain 59% of the variance in HRG levels.

The relationship between HRG plasma levels and thrombosis is investigated in four thrombophilic families of which the probands have been selected for the presence of elevated HRG and thrombosis (chapter 6). Since data obtained from family members are not independent data, statistics specially developed for family studies were used to test

for differences in HRG levels between individuals with thrombosis and unaffected individuals. In these four families (n=99) supplemented with 20 healthy volunteers no difference in mean HRG level is found between individuals with and without thrombosis. In chapter 7 the implications of the present research for the role of elevated HRG levels in thrombosis will be discussed.

Chapter 2

Plasma HRG levels: heritability

Chapter 2.1

A parent-twin study of plasma levels of histidine-rich glycoprotein (HRG)

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Summary

Histidine-rich glycoprotein (HRG) is a non-enzymatic glycoprotein that acts as a modulator of several plasma proteins involved in coagulation and fibrinolysis. The contributions of genetic and environmental influences to inter-individual variation in plasma levels of HRG were studied in 160 Dutch families consisting of adolescent twin pairs and their parents. Results showed that 69% of the variance in plasma HRG concentrations could be accounted for by genetic factors. Heritability was the same in males and females and in parents and their offspring. There was no association between HRG levels of husband and wife and no evidence was found for the influence of a shared family environment on the resemblance between relatives.

Introduction

Histidine-rich glycoprotein (HRG) is a single chain glycoprotein that is found in plasma at a concentration of 125 mg/l (Koide et al., 1986a; Morgan et al., 1978a) and in platelets at approximately 0.07% of plasma HRG concentrations (3). Plasma HRG is produced by the parenchymal cells of the liver (Smith et al., 1989; Hennis et al., 1991a). The source of platelet HRG is uncertain, it may be synthesized by megakaryocytes (Leung et al., 1983). Since the purification of HRG in 1972 (Heimburger et al., 1972) many possible functions have been assigned to the protein and a diversity of biological interactions of HRG have been described. Plasma HRG is able to bind plasminogen (Lijnen et al., 1980), thrombospondin (Leung et al., 1984), and heparin (Koide et al ., 1982). HRG binds to heme (Morgan et al., 1978b; Morgan et al., 1981) and several divalent metal ions also have affinity for HRG. Despite this knowledge, the physiological function of HRG remains unknown.

An important property of HRG is its specific affinity for plasminogen. Around 50% of circulating plasminogen is bound to HRG. When HRG levels are elevated the amount of free plasminogen is reduced and this may cause an inhibition of fibrinolysis since only non-bound plasminogen binds to fibrin (Lijnen et al., 1980). The ability of HRG to bind heparin and plasminogen suggests a procoagulant and antifibrinolytic effect, respectively, and may form a basis for the increased prevalence of elevated HRG levels in patients with venous thrombosis (Engesser et al., 1988). In families with familial thrombosis familial elevation of HRG levels is seen and genetic influences on plasma HRG concentrations in these specific families have been suggested (Engesser et al., 1987; Falkon et al., 1991; Schved et al., 1991).

The cDNA for HRG has been cloned and the gene for HRG has been assigned to chromosome 3 (van den Berg et al., 1990), but as yet the characteristics of the structural gene for HRG are unclear and very little is known about the determinants of individual differences in plasma HRG concentrations in the general population.

In the present study we focus on the contribution of genetic and environmental factors to inter-individual variation in plasma HRG levels in a random sample from the general population. Plasma HRG levels were measured in 160 adolescent twin pairs and their parents to examine the contributions of genetic and environmental factors. In a twin design the separation of genetic and environmental variance is possible because monozygotic (MZ) twins share 100% of their genetic make-up and dizygotic (DZ) twins share on average 50% of their additive genetic variance. If a trait is influenced by genetic factors, MZ twins should resemble each other to a greater extent than DZ twins. When twice the DZ correlation is greater than the MZ correlation, this may indicate that part of the resemblance between twins is induced by the shared family environment (Neale et al., 1992). Parents and offspring also share 50% of their additive genetic variance, as well as a common family environment. By including parents of twins in the design the presence of assortative mating (a correlation between HRG levels of husbands and wifes) and differences between generations in heritability can be examined.

Methods

Subjects

This study is part of a larger project in which cardiovascular risk factors were studied in 160 adolescent twin pairs and their parents. Addresses of twins (between 14-21 years of age) living in Amsterdam and neighboring cities were obtained from City Council population registries. Twins still living with both their biological parents were contacted by letter. A family was included in the study if the twins and both parents were willing to participate. In addition, a small number of families who heard of the study from other twins also volunteered. Zygosity was determinated by typing the following polymorphisms: ABO, MNS, P, Rhesus, Lutheran, Kell, Duffy, Kidd, Gm, Am and Km. Thirty-five twin pairs were also typed by DNA fingerprinting (Jeffreys et al., 1985). Three series of triplets were included by discarding the data from the middle child. There were 35 MZ female pairs (average age 16.0, SD=2.2),35 MZ male pairs (16.6, SD=1.8), 30 DZ female pairs (17.7, SD=2.0), 31 DZ male pairs (17.2, SD=1.7) and 29 DZ unlike sex pairs (16.4, SD=1.8). Average age of fathers was 48.1 (SD=6.3) and of mothers 45.6 (SD=5.9).

Procedure

Fasting blood samples were taken between 8:30 and 10:30 AM. Blood was collected under standardized conditions by venipuncture, using Becton-Dickinson Vacutainers containing sodium-EDTA. Plasma was separated from cells after centrifugation at 3000 rpm for 10 minutes at 4°C. The samples were stored at -20°C and thawed immediately before use. Plasma HRG levels were measured by radial immunodiffusion (Mancini et al., 1965) using 1% agarose plates with 7.5 mM veronal buffer (5,5-diethylbarbturicacid) pH 8.6 and 0.4% home-made rabbit anti HRG-antiserum. Polyclonal rabbit

anti HRG antibodies were raised against purified human HRG kindly provided by Dr. Heimburger (Behringwerke, FRG). Before measuring, plasma was diluted four times in phosphate buffered saline pH 7.4 using an automated diluter (Hamilton Bonaduz AG, Switzerland). Five ml of the diluted sample was placed in a well. Sharply delineated precipitation rings were observed. HRG concentrations were calculated from a standard dilution series of pooled plasma obtained from 20 healthy volunteers. HRG levels were expressed as a percentage of pooled plasma, taking pooled plasma as 100%. All measures were carried out in duplicate and averaged. The inter-assay precision (coefficient of variation) of the duplicate measurements was 10%. Family members were never measured on the same agarose plate in order to exclude the possibility that resemblance between family members is influenced by inter assay fluctuations.

Statistical analysis

The effects of sex, generation and zygosity on mean HRG levels, on variances and correlations between relatives were assessed by likelihood-ratio c^2 tests. These tests were used to compare the fit of a model that constrained parameter estimates to be equal across sexes or generations to one which allowed them to vary, while taking into account the dependency that exists between observations from family members (for a detailed description of the statistical procedures see Boomsma et al. (1993a).

Genetic model fitting was carried out on variance-covariance matrices of the 5 different family groupings (i.e. families of MZ male and female twins and families of DZ male, female and opposite-sex twins). Genetic models specified variation in phenotype to be due to genotype and environment. Sources of variation considered were G, additive genetic variation (i.e. the sum of the average effects of the individual alleles at all loci); C, common environment shared by family members living in the same household and E, a random environmental deviation that is not shared by family members. Their influence on the phenotype is given by parameters h, c, and e that are equivalent to the standardized regression coefficients of the phenotype on G, C and E, respectively. The proportion of variance due to each source is the square of these parameters. To account for possible sex or generation differences in genetic architecture, three different genetic models were examined:

A. full model in which estimates for h, c, and e are allowed to differ in magnitude between males and females, or between parents and offspring,
B. scalar model in which parameters h, c, and e in one sex or generation area constant multiple of the parameters in the other sex or generation. The model allows the total variances for each group to be different, but the relative importance of genetic (heritability) and environmental influences is constrained to be equal across sexes or generations (Neale et al., 1992),
C. constrained model in which 1 or all parameter estimates for h, c and e are constrained to be equal in magnitude across sexes or generations.

Parameters h, c and e were estimated by maximum likelihood, using the computer program LISREL7 (Jöreskog et al., 1988). Goodness-of-fit was assessed by

likelihood-ratio c^2 tests. The overall c^2 tests the agreement between the observed and the predicted variances and covariances in the 5 family groupings. A large c^2 (and a low probability) indicates a poor fit, while a small c^2 (accompanied by a high p-value) indicates that the data are consistent with the model. Submodels were compared by hierarchic c^2 tests, in which the c^2 for the full model is subtracted from that for a reduced model. The degrees of freedom (df) for the this test are equal to the difference between the df for the full and the submodel (Neale et al., 1992). The scalar model (B) is a submodel of the full model (A) and the constrained model (C) is nested under (B).

Results

Table 1A lists means and standard deviations in HRG levels for fathers, mothers, sons and daughters.

Table 1. Maximum likelihood estimates of HRG means and standard deviations (expressed as a percentage of pooled plasma) for fathers, mothers, sons and daughters; c^2 statistics and corresponding probabilities for models of sex and generation differences in means and standard deviations.

A.	Fathers	Mothers	Sons	Daughters
Mean	127.2	120.3	112.5	106.1
SD	32.4	28.8	24.7	30.6
		c^2	df	*p*
B. Test of means				
No differences		136.20	61	0.000
Sex differences		133.82	60	0.000
Generation differences		74.66	60	0.096
Generation and sex differences		65.76*	58	0.226
C. Test of standard deviations				
No differences		52.59	39	0.072
Sex differences		52.58	38	0.058
Generation differences		48.41	38	0.120
Generation and sex differences		39.96*	36	0.299

*preferred model

In table 1B and 1C the results of testing for sex and generation differences in means and standard deviations are presented. Both means and standard deviations differ significantly between sexes and generations. Parents have higher HRG levels than their children and in both generations males have higher HRG levels than females. Parents also are more variable than their children, but whereas fathers are more variable than mothers, in the generation of the children girls are more variable than boys.
Correlations between twins, spouses, and parents and their offspring are summarized in table 2A. It can be seen that MZ correlations are larger than DZ and parent-offspring correlations. Table 2B gives the results of significance testing of familial correlations, by comparing submodels in which correlations are constrained to be zero or in which correlations are constrained to be equal to other correlations. The correlation between husband and wife is low (r=0.12) and not different from zero c^2 difference between the last model in table 1C in which the spouse correlation is free and the model in table 2B in which it is constrained to be zero is 2.38 which is a non-significant increase in c^2.

Table 2. Maximum likelihood estimates of familial correlations for HRG levels in twins, spouses and parents and offspring; c^2 satistics and corresponding probabilities for different models testing equality of correlations.

A.	MZM	MZF	DZM	DZF	DOS	All MZ	All DZ
Correlation	0.69	0.72	0.49	0.45	0.40	0.70	0.43
	Spouses	Fa-Son	Fa-Dau	Mo-Son	Mo-Dau	All Parent-Child	
Correlation	0.12	0.48	0.34	0.34	0.37	0.34	
B. Significance tests		c^2		df		*p*	
Spouse correlation zero		42.34		37		0.251	
MZ correlations equal		42.47		38		0.285	
DZ correlations equal		42.69		40		0.356	
MZ = DZ correlation		50.90**		41		0.138	
Parent-child correlation equal		45.14		43		0.383	
DZ = Parent-child correlation		46.65*		44		0.364	

MZM = Monozygotic Males, MZF = Monozygotic Females, DZM = Dizygotic Females, DZF = Dizygotic Females, DOS = Dizygotic Opposite-Sex Twins. Fa = Father, Dau = Daughter, Mo = Mother.
*Preferred model. **Signifiant increase in c2.

Constraining the 2 MZ correlations to be equal to one another or the 3 DZ correlations to be the same (i.e. specifying them to be equal for males and females) does not lead to significant increases in c^2. However, the test of differences between the MZ and DZ correlations gives a highly significant increase in c^2, indicating that MZ twins resemble each other more than DZ twins.

Parent-offspring correlations can be constrained to be the same (i.e. identical correlations between father and son, mother and son, father and daughter, and mother and daughter) and, finally, the DZ and the parent-offspring correlations can also be equated to each other without a decrease in fit. Estimating one MZ correlation and one correlation for DZ twins and parent and offspring, while constraining the spouse correlation to be zero gives maximum likelihood estimates of 0.69 for the MZ correlation and of 0.34 for the DZ and parent-offspring correlations. These correlations suggest a simple genetic model for inter-individual variation in HRG levels without sex or intergenerational differences in heritabilities. As the DZ and parent-offspring correlations are exactly half the MZ correlation, there also does not seem to be an effect of shared environment.

Table 3. *Genetic model fitting; c^2 statistics and probabilities for different genetic / environmental models of familial resemblance.*

		c^2	df	*p*
A	Sex and Gener differences in G, E, C	40.03	38	0.380
	Sex and Gener Differences in G, E	45.98	42	0.311
	Sex and Gener differences in C,E	53.27	42	0.114
B	Scalar Model, G,E,C	44.38	44	0.456
	Scalar Model, G,E	46.66*	45	0.404
	Scalar Model, C,E	76.72	45	0.002
C	No Sex or Gener Differences in G,E,C	59.18	47	0.110
	No Sex or Gener Differences in G,E	62.14	48	0.083
	No Sex or Gener Differences in C,E	95.47	48	0.000

G represents additive genetic influences, C common environment shared by family members and E individual specific environmental influences. * Preferred model.

Fifteen mothers of twins, and 13 MZ and 11 DZ female twins used oral contraceptives. Although this influences HRG levels, it did not influence familial resemblances very much. When women using contraceptives are excluded from the sample twin correlations are 0.69 for MZF (was 0.72), 0.42 for DZF (was 0.45) and 0.42 for DOS (was 0.40). Genetic analyses were therefore carried out using data from all families. Table 3 presents the results of the genetic model fitting analyses. Models in table 3A that include a genetic component and allow for sex and generational differences in the size of the parameter estimates show a reasonably good fit to the data. Compared to the full G,C,E model a G,E model in which familial resemblance is completely accounted for by additive genetic

factors, can be accepted, as there is a non-significant increase in c^2. Heritabilities under this model are between 55% and 70%. However, when a C,E model is analyzed in which familial resemblance is entirely due to the shared family environment, this leads to a significant increase in c^2 (c^2 difference is 15.24 with 4 df, p=0.004).
Constraining heritabilities to be the same in both sexes and across generations while allowing for differences in variances (scalar model 3B) provides the best fitting model and gives most parsimonious account of the data. This model gives a heritability estimate for HRG levels of 69%. Models that do not take into account the differences in total variances between sexes and generations do not fit the data, although it may be seen in table 3C that a model including a genetic component always gives a better explanation of the data than a purely environmental one.

Discussion

This is the first study to examine the inheritance of plasma HRG levels in an unselected twin sample from the general population. Evidence was found for a simple additive genetic model of inheritance. Correlations between MZ (r=0.70) and DZ twins (r=0.43) and parents and their offspring (r=0.34), as well as the model fitting results indicate that familial resemblance in HRG concentrations can be accounted for by genetic factors. There is no influence of the shared family environment, despite the fact that our sample consists entirely of parents and children living in the same household and to a large extent sharing the same diet (Boomsma, 1990). The amount of inter-individual variance that is due to non-genetic factors (31%) is made up only of environmental influences that are unique to each individual. Genetic factors explain 69% of the variance in plasma HRG levels. The genetic heritability is the same in males and females and in parents and offspring. Twin studies sometimes have been criticized for giving heritability estimates that are higher than the estimates obtained from other family groupings. In our study the resemblances between parents and offspring who share 50% of their genetic material agree very well with the resemblances between DZ twins who also have on average 50% of their genes in common. Comparing parent-offspring and DZ twin correlations may offer a first suggestion about age-dependent genetic effects. Significant parent-offspring resemblance implies significant heritabilities both in adolescence and adulthood and a substantial genetic correlation across time. We found that the parent-child and the DZ correlations for HRG levels do not differ significantly from each other. This implies that there must be substantial stability from adolescence to adulthood in the genetic factors that influence variation in HRG levels, i.e., that the same genes are expressed at different ages.

Other haemostatic factors show a pattern of genetic inheritance that very closely resembles these results for HRG. Hamsten et al. (1987) obtained a heritability of 51% for plasma fibrinogen concentrations in a family study. Iselius (1988) reports a

heritability of 41% for Factor VIII using the same family material. No evidence for shared environmental factors or for intergenerational differences in heritability as found for either fibrinogen or Factor VIII.
It is clear that the familial elevation of HRG levels that is seen for example in some families with familial thrombosis cannot be attributed to common environmental influences shared by family members, but has to be attributed to genetic influences shared by parents and children. The substantial heritability that was found justifies the search for quantitative trait loci, which is possible with sibling (Goldgar, 1990) or twin data (Vogler, 1992).
From our study we cannot draw any conclusions regarding the association between elevated HRG levels and familial thrombosis. Linkage disequilibrium may be involved (Ott, 1995) but several other mechanisms also are possible. It may be that an elevated HRG level favors the development of thrombosis. This would imply a causal mechanism where the genes that influence HRG levels do not themselves directly predispose to thrombosis. Another possible explanation for the association between elevated HRG and thrombosis is pleiotropy, where the genes that influence HRG levels also directly influence thrombosis predisposition. These last two mechanisms can be distinguished from each other with cross-sectional twin data or data from other genetically informative constellations of relatives (Neale et al., 1992; Duffy et al., 1993; Heath et al., 1989) if information on HRG levels and thrombosis status is available for all subjects.

Acknowledgements

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Chapter 2.2

Estrogens reduce plasma histidine-rich glycoprotein (HRG) levels in a dose dependent way

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Thrombosis and Haemostasis: in press.

Summary

Plasma levels of histidine-rich glycoprotein (HRG) were investigated in three groups of women receiving a different dose of estrogens. First, the effect of low-dose estrogen was studied in a group of 83 postmenopausal women who were treated with 0.625 mg conjugated estrogens (CE). No significant change from baseline levels was found at the end of cycle 3 and cycle 13. Secondly, in 15 mothers and 23 daughters using oral contraceptives (OC) containing 30-50 mg ethinyl estradiol (EE) daily the mean HRG level was 14% and 24% lower than in a group of 144 mothers and 134 daughters not taking oral contraceptives, respectively ($p < 0.05$). Finally, in 11 excessively tall prepuberal girls who received 300 mg EE daily to reduce their final height the mean plasma HRG levels were decreased by 68% ($p < 0.005$). The effect of progestogens administered during low-dose and high-dose estrogen therapy appeared to be minor.
The results from these three studies indicate that estrogens reduce plasma HRG levels in a dose-dependent way.

Introduction

A dose-dependent, increased risk of thromboembolism has been reported to be associated with the estrogen component of oral contraceptives (1). In addition there have been some case reports of thrombosis during high-dose estrogen therapy, but the exact incidence is unknown (2,3). The reason for the increased risk is as yet unknown. One of the factors that may play a role is histidine-rich glycoprotein (HRG). Due to its interaction with haemostatic parameters like plasminogen (6), fibrin (7), and heparin (8), HRG is thought to act as a modulator of coagulation and fibrinolysis (4).
In a recent study individual plasma levels of HRG appeared to be very stable in time (16). During a period of 6 months only minor fluctuations were observed in HRG levels of 20 healthy volunteers and no evidence of seasonal fluctuations was found. The overall genetic influence on plasma HRG levels has been determined in a parent-twin study (15). 69% of the variance in HRG levels could be ascribed to genetic factors, the other 31% of the variance could be explained by individual environmental factors. So far only a few factors have been reported which lead to an alteration of the plasma HRG levels. In the majority of the cases a decrease is reported due to diseases like severe liver malfunction (17) and sepsis (18), or to immunosuppressive steroid therapy (19). Only in patients suffering from thrombosis (10,12) or mild (Child A) liver disease (17) is an increase seen. Furthermore HRG levels are negatively correlated with C-reactive protein (CRP) levels, indicating that HRG levels exhibit a negative response in the acute phase (20).
In a few studies the marked effect of female sex hormones on the HRG level has been reported. A significant decrease of HRG levels (17-36%) has been found upon administration of oral contraceptives (21,22). Low levels of HRG have been observed during the third trimester of pregnancy with the lowest levels (40-50% of the normal

level) at parturition (23). A small cyclic fluctuation of HRG levels has been observed during the menstrual cycle with the highest HRG levels during the early follicular phase, in which hormone levels are low (24).
To further evaluate the effect of female sex hormones on the HRG plasma level, we investigated the effect of various doses of estrogen in three different groups. The effect of low-dose estrogen was studied in a group of postmenopausal women receiving hormone replacement therapy. In a large sample of women from the general population the effect of an intermediate-dose estrogen was studied by comparing women using oral contraceptives with non-using women. The effect of high-dose estrogen was studied in prepuberal girls who received hormone therapy to reduce their final height.

Subjects and methods

Low-dose estrogen

Eighty-three postmenopausal women who had undergone hysterectomy were recruited by advertisement and articles in daily newspapers. Women between 50 and 65 years of age were included if serum Follicle Stimulating Hormone (FSH) was > 40 IU/l and serum 17-b-estradiol < 148 pmol/l. Women were excluded if they had used oral estrogens and/or progestogens less than 3 months before the start of the study and if they had a positive history of thromboembolic disorders related to estrogen therapy. Two groups were formed by randomization. One group (CE) of 45 women (mean age ± s.d.: 54.9 ± 3.9) received 0.625 mg of conjugated estrogens (CE, Premarin^{R}) continuously. The other group (CE + MDG) of 38 women (54.8 ± 4.0) received the same dosage of CE continuously plus 5 mg of medrogestone (MDG, Colprone^{R}) the last 12 days of each cycle of 28 days (Premarin^{R} and Colprone^{R} are from Wyeth Laboratories, Hoofddorp, The Netherlands). Blood samples were taken in the morning after an overnight fast using vacutainer tubes containing EDTA as an anticoagulant. Plasma was prepared and stored at -70°C. Samples were thawed only once immediately before measurement. Baseline blood samples were taken within two weeks before the start of the therapy. During medication blood samples were drawn between the 22nd and 28th day of the 3rd and 13th cycle.

Intermediate-dose estrogen

HRG levels of a large group of mothers and their daughters were measured in a parent-twin study previously described by Boomsma et al. (15). Mean ages (± s.d.) of mothers (n = 159) and daughters (n = 157) were 45 (± 5.4) and 17 (± 2.2), respectively. 9 mothers and 17 daughters used oral contraceptives with 30 mg ethinyl estradiol, 6 mothers and 6 daughters used oral contraceptives with 50 mg ethinyl estradiol. 144 mothers and 134 daughters did not use oral contraceptives.

High-dose estrogen

Eleven healthy prepuberal girls (mean age ± s.d.: 12.5 ± 1) were treated with high-dose estrogen to reduce their expected final height. They received 0.3 mg ethinyl estradiol daily and 5 mg of medroxyprogesteron-acetate every fourth week daily. Baseline blood samples were taken within one month before the start of the therapy. During medication blood samples were drawn once between 3-7 months after the start of therapy. 9 volumes blood were collected in vacutainer tubes containing 1 volume 3.2% sodium citrate as an anticoagulant. Plasma was prepared and stored in 0.2-0.5 ml aliquots at -70°C. Samples were thawed only once immediately before use.

HRG measurement

Measurement of plasma HRG levels was performed by radial immunodiffusion (25) using 1% agarose plates with 7.5 mM veronal buffer (5,5-diethylbarbituric acid), pH 8.6 and 0.4% rabbit anti-HRG-antiserum. Rabbit polyclonal anti-HRG-antibodies were raised against purified human HRG kindly provided by Dr. N. Heimberger (Behringwerke, Germany). Plasma samples were diluted 1:4 in phosphate-buffered saline, pH 7.4, using an automated diluter (Hamilton Bonaduz A.G., Switzerland). Five ml of the diluted sample was placed in a well and allowed to diffuse at 4°C for 48 hours. After washing with 0.9% sodium chloride for 48 hours at 4°C, sharply delineated precipitation rings were observed. HRG concentrations were calculated from a standard dilution series of pooled plasma obtained from 26 healthy volunteers. Calibration lines were prepared both from EDTA and citrated pooled plasma to circumvent differences in measurements due to the use of EDTA or sodium citrate as anticoagulant in the different groups. HRG levels were expressed as a percentage of pooled plasma, taking citrated pooled plasma as 100%. The inter-assay coefficient of variation of the duplicate measurements was 10%.

Statistical analysis

For all groups mean HRG levels and standard deviations (s.d.) or standard errors of the mean (s.e.m.) were calculated. ANOVA was used to test for differences in HRG level between the two treatment groups in the low-dose estrogen group and between users and non-users of OC in the intermediate-dose group. Differences in age and baseline HRG levels between groups were also tested with ANOVA. Repeated measures ANOVA was used to test for differences across time in the low-dose and high-dose estrogen group.

Results

Effect of low-dose estrogen

HRG plasma levels were determined in two groups of hysterectomized postmenopausal women receiving hormone replacement therapy to evaluate the effect of low-dose estrogen. Mean HRG levels and ranges are shown in Table 1. Baseline HRG levels of

the two treatment groups were not statistically significant. During treatment no significant change across time was observed both in the group receiving CE and in the group taking CE + MDG. The difference in response between the groups was also not statistically significant.

Table. 1. Effect of low-dose estrogen treatment on plasma HRG levels in hysterectomized postmenopausal women.

	Mean % HRG (s.e.m.)		
Group	Baseline	3 cycles	13 cycles
CE (n=45)	113 (3.3)	111 (3.5)	112 (3.6)
Range	69-174	66-171	68-177
CE + MDG (n=38)	106 (3.2)	106 (3.3)	104 (3.3)
Range	74-163	75-163	79-164

HRG levels and s.e.m. are expressed as a percentage of pooled plasma. CE: group recieving conjugated estrogen. CE + MDG: group recieving conjugated estrogen plus medrogestone.

Effect of intermediate-dose estrogen

The effect of intermediate-dose estrogen was determined by comparing HRG levels of women using oral contraceptives to non-using women in a group of mothers and their daughters. Mean HRG levels are shown in Table 2. Mothers and daughters not using OC have significantly different mean HRG levels indicating an effect of age (see also Table 4).

Table. 2. Mean HRG levels and range of mothers and daughters. Effect of oral contraceptives.

	No OC	30 or 50 mg EE	30 mg EE	50 mg EE
Mothers (n)	144	15	9	6
Mean HRG (s.e.m.) %	102 (1.7)	88(5.8)($p<0.05$)[#]	87 (7.3)	89 (8.7)NS[*]
Range	58-168	43-119	50-119	43-119
Daughters (n)	134	23	17	6
Mean HRG (s.e.m.) %	93 (1.8)	71 (4.6)($p<0.001$)[#]	75 (5.9)	62 (5.0)NS[*]
Range	49-168	36-118	36-118	46-76

[#] P value of an unpaired t-test between the mean level of mothers or daughters using OC versus not using OC. [*] P value of an unpaired t-test between users of OC containing 30 mg EE and 50 mg EE. NS: not significant.

Differences between users of OC and non-users were therefore tested separately in mothers and daughters. Mothers ($n = 15$) using 30-50 mg EE have on average 14% lower HRG levels than non-using mothers ($n = 144$) ($p < 0.05$). The mean HRG level

in daughters (n = 23) using 30-50 mg EE is 21% lower ($p < 0.001$) than in non-users (n = 134). Women taking OC can be divided in groups using OC containing 30 or 50 mg EE. Daughters using 50 mg EE tend to have a lower HRG level than daughters using 30 mg EE but this difference is not significant. In mothers there was no difference between users of 30 and 50 mg EE.

Effect of high-dose estrogen

The effect of 300 mg EE on the HRG level has been evaluated in 11 prepuberal girls. The mean HRG level was lowered from 90% at baseline to 29% during therapy, which is an average decrease of 68% ($p < 0.005$) (Table 3). No effect was observed from differences in duration (3-7 months) of the therapy (data not shown).

Table 3. Mean HRG levels and range of 11 adolescent girls treated with high dose estrogen.

	Baseline	During therapy
Mean HRG (s.e.m.) %	90 (6.2)	29 (3.9) ($p=0.005$)*
Range	63 - 133	8 - 48

* $p<0.05$ in a repeated measures ANOVA

Effect of age on baseline HRG levels

The effect of age on HRG levels was studied using the baseline HRG levels of postmenopausal women, prepuberal girls and mothers and daughters not using OC. Mean ages and baseline HRG levels of the three groups are shown in Table 4. The mean age of the two treatment groups of postmenopausal women was not significantly different. Therefore the baseline levels of the two groups were combined.

ANOVA among the groups investigated revealed significant differences in age and mean HRG levels (Table 4). HRG levels tend to become higher as the age of the group studied increases: prepuberal girls (mean age = 12.5 yrs) and daughters (16.2 yrs) had about 10% lower HRG levels than mothers (46 yrs) ($p < 0.05$). Postmenopausal women (55 yrs) had on average 8% higher HRG levels than mothers ($p < 0.05$).

Table 4. Baseline HRG levels and age.

Group (n)	Age (s.e.m.)	Mean % HRG (s.e.m.)
Postmenopausal women (83)	55.0 (0.43)	110 (2.3)
Mothers (144)	46.0 (0.49)	102 (1.7)
Daughters (134)	16.2 (0.16)	93 (1.8)
Prepuberal girls (11)	12.5 (0.30)	90 (6.2)

Differences in age and mean HRG levels were tested using ANOVA. Except for the difference in HRG level of prepuberal girls versus daughters and prepuberal girls versus mothers, all differences both in age and HRG levels between the four groups were significant ($p < 0.05$).

Discussion

The results of our study show a dose-dependent influence of estrogen on the HRG plasma level. During low-dose estrogen treatment in hormone replacement therapy, no effects on the HRG level were found. The use of oral contraceptives was associated with a decrease of the HRG level by 14-24% and the administration of high-dose estrogen leads to a lowering of the level by 68%. In clinical terms, a dosage of 625 mg of conjugated estrogens is comparable to 5 mg of ethinyl estradiol. Thus the low-dose estrogen group received the equivalent of 5 mg ethinyl estradiol while the oral contraceptive group received 30-50 mg and the high-dose estrogen group 300 mg.
A point that should be mentioned is that of the presence of different progestogens in the hormone preparations. In general progestogens are added during the estrogen treatment in a dosage just enough to prevent endometrial adenocarcinoma (26). In our study the dose of medrogestone received by the low-dose estrogen group was similar to that of medroxyprogesteron acetate in the high-dose estrogen group because both progestogens are derivatives of 17-hydroxy-progesterone and are thought to have comparable physiological effects (27). Since no effect of treatment on the HRG level was observed in the low-dose estrogen group, whereas a dramatic effect was observed in the high-dose estrogen group, it can be assumed that the progestogen component has a negligible influence on the HRG level. This is in support with the observations of Haukkamaa et al. (23), who reported that the subcutaneous administration of 60 mg/day of Progestin ST-1435 had no effect on the HRG level.
The groups in the present study differ markedly with respect to age and baseline HRG levels. HRG levels tend to increase with increasing age. A part of the age effect found in this study has previously been described by Boomsma et al. (15). They reported a generation effect in a parent-twin study. Both fathers and mothers have higher HRG levels than their sons and daughters, respectively.
In the present study, the increase in HRG seemed not to be linear with respect to time since an increase of 9% is observed in the 30 years between daughters and mothers whereas an increase of 8% is found in the 9 years between mothers and postmenopausal women. This may indicate an effect of the menopause. A related observation was done by Thompson et al. (28), who found that the increase of HRG was greater in women than in men after the age of 50. These results suggest a small age effect which is probably also present in men but in addition to this there is possibly also an effect of the menopause on the HRG level.
Whether the magnitude of the effect of estrogens is also age-dependent, remains unclear. Due to the design of the study it is not possible to draw any conclusions in this field.

From previous studies (21,22) and our own study it is obvious that the administration of exogenous estrogens reduces HRG levels. Several results indicate that there is also a relationship between endogenous oestradiol levels and HRG. Men have higher HRG levels than women (15), but this difference is reversed after the menopause (28) when

oestradiol levels in women are decreased. HRG levels are low both during the last trimester of pregnancy (23) and day 12-16 of the menstrual cycle (24), when oestradiol levels are increased. However, no correlation between serum HRG and total serum oestradiol was found in a cross-sectional study in women (23). Therefore, the possible relationship between endogenous oestradiol levels and plasma HRG levels needs to be studied in further detail.

Plasma HRG is synthesized by the liver (5) and although deviating HRG levels are observed mainly under circumstances with aberrant liver function (17), little is known about the regulation of the HRG level. Both conjugated estrogens and ethinyl estradiol have rather strong effects on several haemostatic parameters synthesized by the liver (22,29). It can therefore be speculated that estrogens reduce HRG levels via an alteration of the liver function. Whether this reduction is caused by reduced synthesis or increased clearance is as yet unclear.
In several studies, elevated levels of plasma HRG have been observed in groups of patients suffering from thrombosis (9-11) and, inherited elevation of HRG has been found in families with thrombosis (12-14). In addition, from *in vitro* experiments it has been suggested that elevated levels of HRG may inhibit plasminogen activation (6). However, the generation of plasmin is not modified in patients with high HRG levels (13) and up to now no evidence has been found for a causal relationship between high plasma HRG levels and thrombosis. The results from the present study show that a dose-dependent reduction of HRG plasma levels during treatment with female sex hormones is caused by the estrogen component. It seems therefore unlikely that plasma HRG levels are related to the increased risk of thromboembolism during estrogen treatment.

Acknowledgements

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Chapter 3

Mapping of HRG locus

Chapter 3.1

Genetic markers at the HRG locus

3.1.1 KPN I RFLP in the human histidine-rich glycoprotein gene

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Source and Description: Probe BCH contains a 2.1 kb cDNA copy of the human HRG gene inserted in pGEM-I (Koide et al., 1986a).

Polymorphism: After Kpn I digestion of genomic DNA the probe detects two allelic fragments of 19.3 kb and 16.8 kb.

Frequency: Estimated from 24 unrelated Caucasians:
19.3 kb allele (A1) 0.61
16.8 kb allele (A2) 0.39.

Not Polymorphic For: AluI, BclI, DraI, EcoRV, HaeIII, HincII, MboII, NdeI, NsiI RsaI, SacI, SspI, StyI, XbaI, XmnI and see: van den Berg et al., (1990).

Chromosomal Localisation: Chromosome 3 (van den Berg et al., 1990).

Mendelian Inheritance: Co-dominant segregation demonstrated in 4 families (25 informative meioses).

Probe Availability: The CDNA of HRG was a generous donation from Dr. T. Koide, Dept. Biochemistry, Niigata University School of Medicine, Niigata 951, Japan.

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3.1.2 PCR detection of a dinucleotide repeat in the human histidine-rich glycoprotein (HRG) gene

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Source and Description: Oligonucleotide primers were used to amplify a 233-267 bp CT and CA repeat-rich region in intron G of the human histidine-rich glycoprotein gene (Koide et al., 1988a).

PCR-Primers:

HRG-CA2F: 5'AAG CAG ACT TTG TCA TGG CAG TGC 3'.

HRG-CA2R: 5' TTG CAC TCC TTT CCC CAG TTG TGG 3'.

Frequency: Fifteen alleles were observed in 153 unrelated individuals. Observed heterozygosity = 0.82.

Allele	bp	Frequency	Allele	bp	Frequency
B1	267	0.01	B9	249	0.08
B2	263	0.01	B10	247	0.01
B3	261	0.01	B11	245	0.10
B4	259	0.05	B12	243	0.17
B5	257	0.02	B13	241	0.01
B6	255	0.06	B14	239	0.37
B7	253	0.01	B15	233	0.06
B8	251	0.03			

Mendelian Inheritance: Co-dominant inheritance was observed in 3 three-generation families and 1 two-generation family.

Chromosomal Localization: Chromosome 3q21-3qter (van den Berg et al., 1990; Naylor et al., 1991).

Other Comments: PCR was performed in a volume of 15 ml containing 30 ng genomic DNA; 30 ng of each primers; 200 mM dATP, dTTP, dGTP; 25 mM dCTP; 0.75 mCi of $a^{32}P$ dCTP (3000 Ci/mmol); 1 x polymerase buffer (Amersham, U.K.) and 0.4 units taq polymerase (Amersham, U.K.). Thermocycling conditions were: 1 min at 94°C, 2 min at 55°C, 1 min at 72°C for 30 cycles. The PCR products were analysed on a 6% denaturing polyacrylamide gel.

Acknowledgements: This work was supported by grant 89.004 from the Dutch Thrombosis Foundation.

Chapter 3.2

Evidence for the absence of intron H of the histidine-rich glycoprotein (HRG) gene; genetic mapping and *in situ* localization of HRG to chromosome 3q28-q29

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Histidine-Rich Glycoprotein (HRG) belongs to the cystatin superfamily (Rawlings et al., 1990) and appears to be a potential risk factor for thrombosis. An increased prevalence of elevated HRG plasma levels in patients with venous thrombosis and families with thrombophilia has been reported (Engesser et al., 1988). It is interesting to note that the genes of 4 different members of the cystatin superfamily are located on the distal section of the long arm of chromosome 3: Stefin A (STF1) on 3q21, Kininogen (KNG) on 3q26-qter, a-2-HS-glycoprotein (AHSG) on 3q27-q28 and HRG on 3q21-qter. To further investigate the evolutionary relationship between HRG and members of the cystatin superfamily we isolated a cosmid which was used to refine the chromosomal localization of HRG by *in situ* hybridization. In addition we used a dinucleotide repeat polymorphism (Hennis et al., 1992) to localize HRG on the linkage map of chromosome 3q.

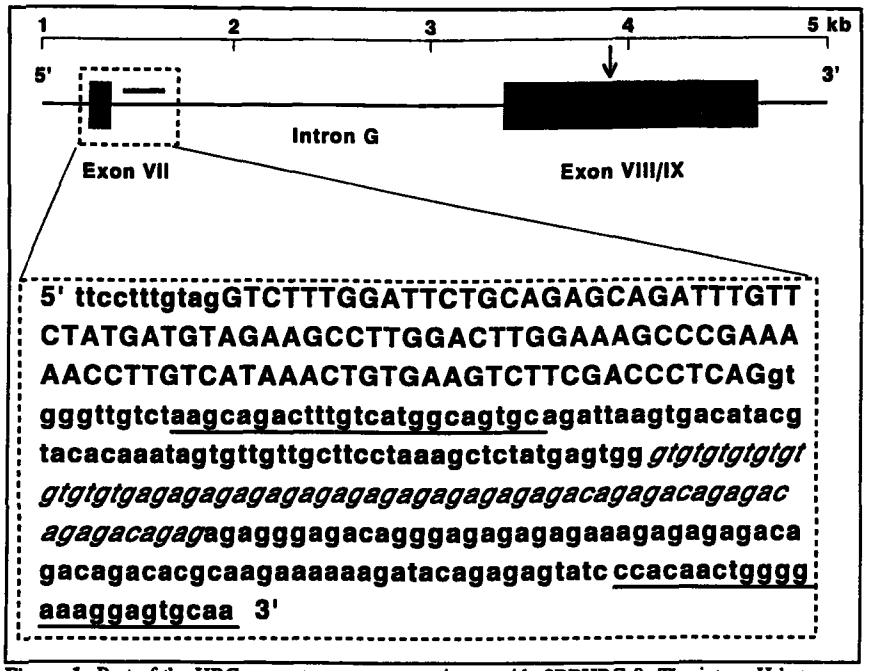

Figure 1. Part of the HRG gene-structure present in cosmid c2RBHRG-8. The intron H between exons VIII and IX proposed by Koide (1988a) is indicated by an arrow. Primers used to amplify the predicted intron H are indicated by arrows below exon VIII/IX. The compound repeat is indicated by a line in the dashed box. The enlargement of the dashed box shows the sequence of the repeat (GenBank Accession No. Z17218) in detail. Sequence identical to exon VII (Koide et al., 1986a) is shown in capitals and the microsatellite repeat sequence is shown in italics. Oligonucleotide primers used to amplify the repeat are underlined.

Using a cDNA (Koide et al., 1986a) probe for HRG, a 45kb cosmid (c2RBHRG-8) was isolated from a cosmid library constructed from DNA of a 49,XXXXY lymphoblastoid cell line. By partial sequence analysis, the presence of exon VII-IX could be confirmed. The sequence of exon VII and of the coding part of exon IX, were found to be identical

to the cDNA sequence reported by Koide et al. (1986a). We also found the same intron-exon boundaries for exon VII as proposed by Koide (1988a). However, by sequencing the predicted boundary between exon VIII and IX, we found no intron H in this genomic clone. The absence of intron H was confirmed by PCR analysis of genomic DNA using primers chosen in exon VIII and exon IX, that amplify the predicted boundary between these exons. Genomic DNA was obtained from freshly collected blood from Dutch volunteers as described previously (Wijmenga et al., 1990). PCR was performed in a volume of 50 ml containing 1 mg genomic DNA, 200 ng of a 5'-primer (5'-CAT GCC ACT TTT GGC ACA AAT GGG-3') in exon VIII and 200 ng of a 3'-primer (5'-TTA TTT TGG AAA TGT ATG TGT AAA AAA CAT GG-3') in exon IX, 200 mM dNTP, 1 x polymerase buffer (Amersham, U.K.), and 0.5 units *Taq* Polymerase (Amersham, U.K.). Thermocycling conditions were 1 min at 94°C (denaturation), 1 min at 55°C (annealing), and 2 min at 72°C (extension) for 30 cycles. In genomic DNA of 40 unrelated individuals, no intron was found. This finding is in contrast to the intron localization proposed by Koide (1988a). They proposed an intron between the codons for amino acids 439 and 440 in the gene for HRG (figure 1).

Cosmid c2RBHRG-8 was used for hybridization to metaphase chromosome spreads. Labelling, hybridization, washing and staining conditions were as described by Wijmenga et al. (1991). Positive hybridization signals were found on the terminal region of the long arm of chromosome 3 at q28-q29. No additional spots on chromosome 3 nor on other chromosomes were seen (figure 2).

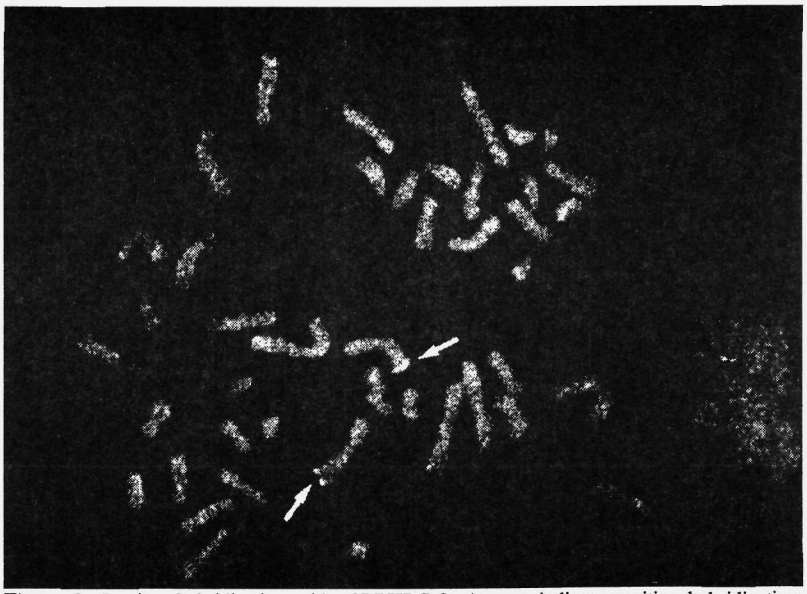

Figure 2. *In situ* hybridization with c2RBHRG-8. Arrows indicate positive hybridization signals.

Clone c2RBHRG-8 was also used to identify CA-repeat regions. A highly polymorphic $(GT)_9(GA)_{13}(CAGAGA)_4$-compound repeat was found in intron G, 93 bp 3' of exon VII (figure 1). The repeat was used to perform linkage analysis on HRG in 40 CEPH reference families. Amplification was carried out as described before (Hennis et al., 1992). Additional marker data were obtained from the CEPH Genotype Database v6.0. Linkage was performed using the programme CRI-MAP 2.4. Information from 29 markers on chromosome 3q from the CEPH Database was used to insert HRG into the linkage map between the loci D3S1427 and D3S1294 (with odds for order at least 1000:1) (figure 3). The interlocus distances between D3S1427, HRG and D3S1294 were 13.5 and 17.8 cM in the female map and 4.7 and 2.7 cM in the male map, respectively. The D3S1262 locus, one of the Genethon markers (Weissenbach et al., 1992), haplotypes with HRG. The observed localization of HRG by *in situ* hybridization and the calculated order on the linkage map are in good agreement with the cytogenetic localization of marker D3S1427 on 3q27 (Hino et al., 1993). The polymorphic marker in the HRG locus, mapping to the most distal band q28-q29 of chromosome 3, provides a PCR marker with a PIC of 0.80, which is useful for filling in a gap in the linkage map near the telomere.

Apart from the homology between the cystatin-like segments and the homology between the histidine-rich region of Kininogen and HRG, the evolutionary relationship between HRG and Kininogen is even more pronounced when the structures of their genes are compared. The intron localization of the two cystatin domains of HRG is very similar to the first two cystatin domains of Kininogen. Moreover, as a consequence of the absence of intron H, the entire region which is situated C-terminal to the cystatin domains of HRG is encoded by a single exon. This is comparable to the 3'-exon of the high molecular weight form of Kininogen (HMWK). In this splice variant of Kininogen, the region which is situated C-terminal of the cystatin domains is also encoded by one exon (Kitamura et al., 1985). In both HRG and HMWK this 3'-exon represents the histidine-rich regions of the proteins.

The genes of two other members of the cystatin superfamily of cysteine protease inhibitors have also been assigned to the distal part of chromosome 3q: KNG (3q26-qter) and AHSG (3q27-q28). In addition to the homologous gene structure, the physical and genetic localization of HRG close to the genes for KNG and AHSG substantiates the evolutionary relatedness of HRG to these members of the cystatin superfamily. Elucidation of the physiological function of HRG might help in understanding the homology between HRG and members of the cystatin superfamily. The availability of a PCR based genetic polymorphism of HRG will be useful for the study of the pathophysiological role of HRG in families with thrombosis.

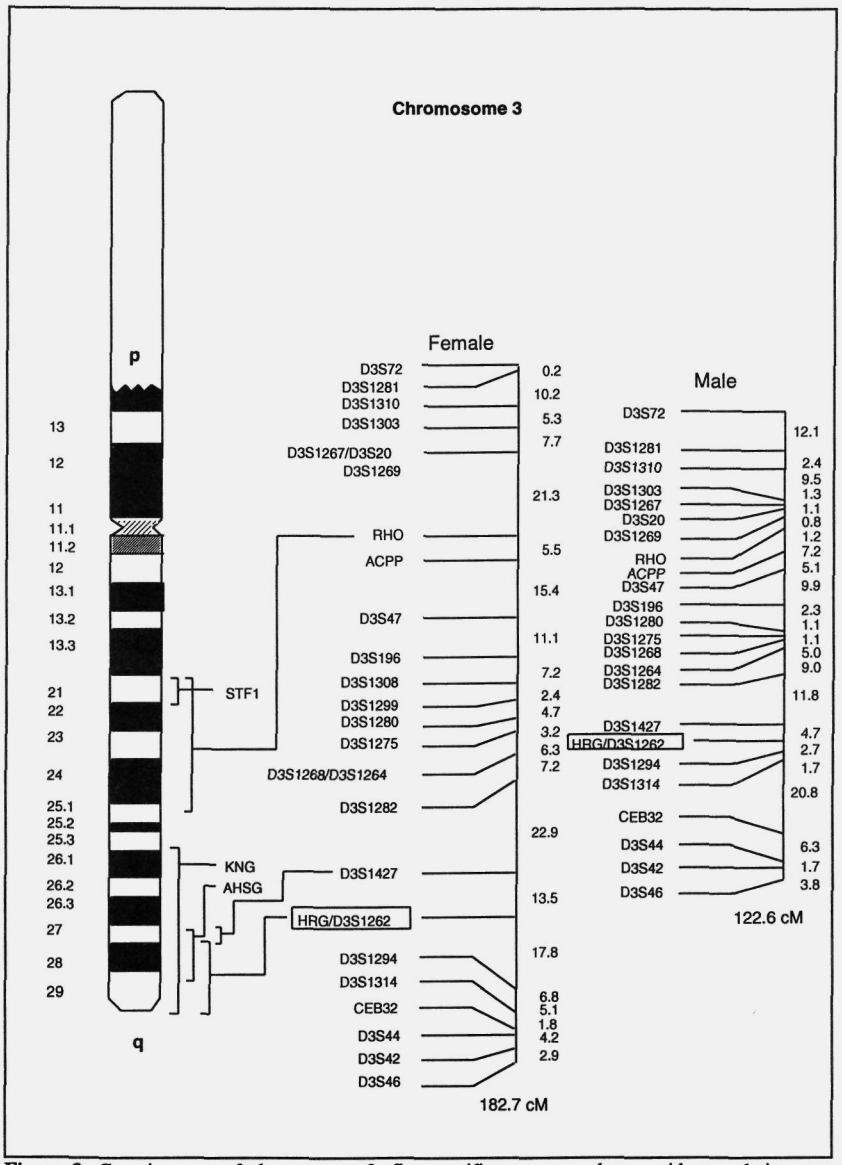

Figure 3. Genetic maps of chromosome 3. Sex-specific maps are shown with cumulative map lengths indicated at the bottom of each map. Distances were computed using the Kosambi mapping function. Not shown on the male linkage map are probes D3S1308 and D3S1299 which haplotype together with probe D3S196. Cytogenetically localized loci (Rhodopsin (RHO) (Sparkes et al., 1986), Stefin A (STF1) (Naylor et al., 1991), Kininogen (KNG) (Cheung et al., 1992) and a-2-HS-Glycoprotein (AHSG) (Magnuson et al., 1988) and D3S1427 (Hino et al., 1993) are indicated on the physical map of chromosome 3.

Acknowledgements

The authors thank Dr. Lodewijk Sandkuijl and Dr. Cisca Wijmenga for linkage analysis, Claus van Leeuwen and Cor Breukel for technical assistance, Dr. Takehiko Koide for the cDNA of HRG and Dr. K. Tartof and Dr. G. Vergnaud for markers. This work was supported by grant 89004 of the Dutch Thrombosis Foundation and by the Eurogem project which is funded by the E.C. Human Genome Analysis Programme.

Chapter 4

Molecular variants and polymorphisms of HRG

Chapter 4.1

Hereditary increase of plasma histidine-rich glycoprotein associated with abnormal heparin binding (HRG Eindhoven)

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Summary

Plasma histidine-rich glycoprotein (HRG) was found to be persistently increased in a patient with a history of recurrent arterial thromboembolic events. The mean concentration was 270% of normal pooled plasma. Increased HRG was found in eight of the 17 relatives studied, but none of them has experienced thromboembolism yet. Apparently, increased HRG was hereditary with autosomal dominant inheritance. A significant correlation was found between the increased plasma concentration of the protein and the age of the subjects ($p<0.02$), whereas no such relation is present in a normal population.
The plasma HRG of the proposita and nine of her family members displayed abnormal binding to heparin, as assessed in a crossed affinity immuno-electrophoresis system: the usual increase in mobility after binding to heparin was absent. The binding of this variant HRG to plasminogen was normal. This case represents the first abnormal HRG variant reported and it is proposed to designate it: HRG-Eindhoven.

Introduction

Histidine-rich glycoprotein (HRG) is an a_2-globulin which was first isolated from human plasma in 1972 (Heimburger et al., 1972) and whose primary structure has been established (Koide et al., 1982; Koide et al., 1986a). HRG is a single-chain glycoprotein with a high content of histidine and proline; it contains about 14% carbohydrate groups and its molecular weight is approximately 68 kDa (Koide et al., 1986a). The amino acid sequence of HRG, derived from its cDNA, indicates a high degree of homology with other coagulation proteins. The C-terminal part of HRG is similar to histidine-rich sequences in high molecular weight kininogen (Koide et al., 1986a), while in its N-terminal part HRG shares homology with the high-affinity heparin binding sites of antithrombin III (Koide et al., 1982). The physiological function of HRG is not yet known, but a number of properties, potentially relevant for haemostasis have been found. For instance, HRG can bind to heparin (Heimburger et al., 1972; Lijnen et al., 1983a; Lijnen et al., 1984), to plasminogen (Lijnen et al., 1980; Ichinose et al., 1984), to fibrinogen and fibrin (Leung, 1986) and to activated platelets (Lerch et al., 1988).

As a consequence of HRG binding reversibly to plasminogen, the concentration of free plasminogen available for activation will be reduced and therefore HRG can be regarded as an antifibrinolytic or prothrombotic protein (Lijnen et al., 1980). Theoretically, high plasma concentrations of HRG might predispose to thromboembolic complications and indeed a number of patients with thrombophilia have been described who had persistent, sometimes familial, increase in their plasma HRG (Castel et al.,1983; Samama et al., 1983; Engesser et al., 1987; Falkon et al., 1992; Anglés-Cano et al., 1993). To date,

however, there is insufficient evidence for presuming a causal role of HRG in the development of thromboembolic disorders.
A few families have been reported with hereditary abnormalities of HRG; they were all characterized by increased plasma concentrations of apparently normal HRG (Engesser et al., 1987; Falkon et al., 1992; Anglés-Cano et al., 1993; Engesser et al., 1988; Shigekiyo et al., 1992). We now describe another family with hereditary, increased HRG levels in several family members over three generations. Apart from the increase in HRG we found indications of a molecular abnormality in HRG, expressed as impaired heparin binding, in this family. It represents the first abnormal HRG variant described.

Materials and methods

Blood collection

Plasma was obtained by centrifugation (3000g for 15 min at 4°C) of citrate-anticoagulated blood (final citrate concentration 0.01 M), which had been collected by a clean venepuncture after minimal stasis; the first few millilitres were discarded. Plasma was snap-frozen in liquid nitrogen, stored at -70°C and thawed only once. Normal plasma was prepared similarly from blood donated by 46 healthy volunteers, 23 males and 23 females, who were not taking any medication. By definition, the pooled normal plasma contained 1.00 U/mL of all factors for which no international standard preparation was available.
Platelets were isolated from blood collected in 0.1 volume of citrate-theophylline-adenosine-dipyridamole mixture (CTAD tubes; Becton Dickinson, Mountain View, CA, USA) by low speed centrifugation (180g for 5 min at 4°C). The platelets were centrifuged to a pellet, the plasma was removed and the platelets were washed once with Tris-saline-EDTA buffer (Leung et al., 1983), containing CTAD. A platelet lysate was prepared by three freeze-thawing cycles.

Coagulation and fibrinolytic assays

Clotting tests were performed using an automated centrifugal analyzer (ACL-300; Instrumentation Laboratory) and commercial reagents for activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen and all clotting factors. The activities of antithrombin III, plasminogen, a_2-antiplasmin, protein C and plasminogen activator inhibitor (PAI) were determined using established chromogenic substrate methods (Kabi, Stockholm, Sweden and Biopool, Umeå, Sweden). Antigen concentrations of von Willebrand factor (vWF), protein S and plasminogen were measured according to Laurell (Laurell, 1966) or using an ELISA with commercial reagents. Heparin cofactor II antigen was determined by Professor R. Bertina (Dept. of Coagulation Research, University of Leiden, the Netherlands) as described (Bertina et al., 1987). The activity of tissue-type plasminogen activator (tPA activity) in the euglobulin fraction of plasma was measured according to Verheijen et al. (1982), tPA

antigen using a commercial ELISA kit (Biopool) and urokinase-type plasminogen activator (uPA) antigen according to Binnema (Binnema et al., 1986).

Assays of HRG

We used rocket electrophoresis for determining HRG concentrations in plasma (Laurell, 1966); a specific rabbit antiserum to HRG was obtained from Behringwerke (Marburg, Germany). The concentration of HRG was expressed relative to normal pooled plasma which by convention contains 1.00 U/mL. HRG in platelet lysate was measured using an in-house ELISA method and polyclonal rabbit antiserum to human HRG (P. Los and B. Hennis, unpublished).

Qualitatively, HRG was studied using crossed affinity immunoelectrophoresis for investigating the binding properties of HRG (Kluft et al., 1988). In this system, the agarose gels in the first dimension contained 0.5 mM of purified glu-plasminogen or 100 U/mL heparin, as indicated. The heparin preparation used was either standard heparin (Liquemin®; Roche, Basel, Switzerland) or low-molecular weight heparin (Fragmin®; Kabi). In some experiments, EDTA (10 mM) was incorporated into the gel and the buffer. In the second dimension, the gel contained anti-HRG antiserum (0.5%, v/v). After washing and drying, the gels were stained with Coomassie blue. All electrophoreses were carried out under strictly standardized conditions.

Binding of HRG to solid-phase bound heparin was studied by affinity chromatography: plasma was passed over a column of heparin-Sepharose (Sepharose CL-6B; Pharmacia, Uppsala, Sweden) and protein was eluted with a gradient of buffered saline (0 - 1.5 M NaCl in 20 mM Tris, pH 7.4). The HRG content of the fractions collected was determined as stated above. The effect of heparin on the thrombin time of normal and proposita's plasma was investigated as described by Lijnen et al (Lijnen et al., 1983).

Results

Case report

The proposita, a 64 year old woman, came to our attention when she participated in a prospective, multicentre trial investigating haemostatic and fibrinolytic parameters in patients with stable angina pectoris (Thompson et al., 1991). At that moment, she had an extremely high HRG concentration in her plasma, namely 2.70 U/mL (reference range see below). She was known to have long-term hypertension and was obese. Her medical history included a cerebrovascular accident and a left carotid artery thrombosis at the age of 57. During the next year, unstable angina pectoris developed for which she had a coronary bypass graft operation after a failed coronary angioplasty. At 63 years of age, she experienced amaurosis fugax, despite adequate oral anticoagulant therapy. Subsequently, diabetes mellitus was found and recently she had a successful angioplasty of the right coronary artery. During the last two years, she has experienced no further thrombotic events or cardiac complications.

On six occasions over more than four years, her plasma HRG concentration was found to be highly increased: the values ranged between 2.55 and 2.90 U/mL. The platelet HRG content of the proposita was 590 ng/10^9 platelets. Results of comprehensive determinations of coagulation and fibrinolytic parameters are shown in table 1; there was no abnormality detected in the clotting factors II-XI (data not shown).

Table 1. Haemostatic parameters of proposita.

	Result	Reference range
APTT	30.5	20.5-30.0 s
PT	10.2	9.5-12.0 s
TT	10.5	10.5-13.5 s
Fibrinogen (Clauss)	3.9	1.5-4.0 g/l
Factor VIII C	1.45	0.6-1.5 U/ml
von Willebrand factor antigen	1.55	0.6-1.5 U/ml
Antithrombin III activity	1.10	0.80-1.15 U/ml
Protein C activity	1.02	0.75-1.20 U/ml
Protein S antigen	0.92	0.75-1.25 U/ml
Heparin cofactor II antigen	2.00	0.62-1.85 U/ml
Factor XII	0.70	0.60-1.30 U/ml
Euglobulin clot lysis time	> 300	> 180 min
t-PA activity	0	0-100 mIU/ml
t-PA antigen	6.4	3.0-10.0 ng/ml
PA inhibitor activity	30.4	< 15.5 IU/ml
Plasminogen activity	1.20	0.70-1.30 U/ml
Plasminogen antigen	1.42	0.75-1.20 U/ml
a_2-Antiplasmin activity	0.93	0.75-1.20 U/ml
u-PA antigen	2.6	1.30-6.30 ng/ml
Plasma HRG	2.70	0.65-1.59 U/ml
Platelet HRG	590	237-487 ng/10^9plats

Reference range for HRG

In a control population of 111 healthy subjects, aged between 30 and 72 years, the distribution of plasma HRG was not normal, but slightly skewed (figure 1). After logarithmic transformation, mean HRG was 0.99 U/mL and the 2 SD range 0.60 - 1.62 U/mL. The median HRG concentration was 1.00 U/mL with 2.5 and 97.5 percentile values of 0.68 and 1.57 U/mL, respectively. We therefore regarded plasma HRG concentrations ^{3} 1.60 U/mL as increased in our population. There were no indications of sex- or age-dependency apparent.

Mean ($\pm$ SD) concentration of platelet HRG was 362 $\pm$ 63 ng/10^9 platelets in a reference group of 20 healthy individuals.

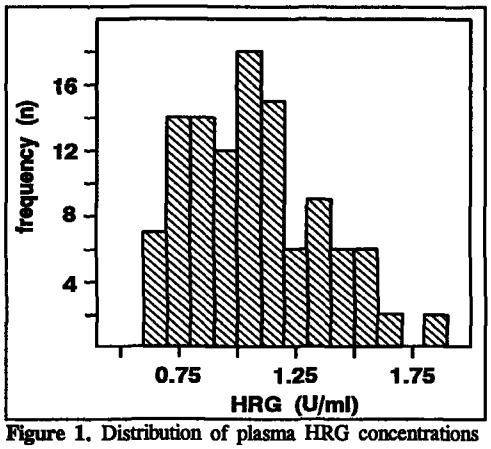

Figure 1. Distribution of plasma HRG concentrations in the healthy reference group (n=111).

Family investigation

The kindred of proposita available for a family study, comprised 17 subjects from three generations. We found an increased plasma HRG in eight of them (table 2 and figure 2). According to their family physicians, none of these individuals had ever been diagnosed as having a thromboembolic disease.

Among the family members with increased plasma HRG there appeared to exist a statistically significant correlation between HRG concentration and the age of the subjects, as shown in figure 3 ($r=0.733$; $p<0.02$ by Spearman ranked correlation test, or $r=0.771$; $p=0.04$ when omitting the three subjects with increased, but qualitatively normal HRG). Such a correlation was not present in our reference population (data not shown).

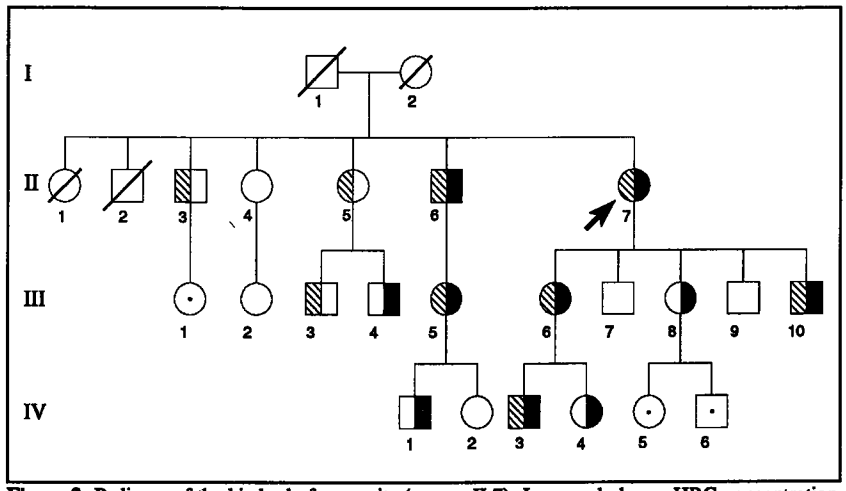

Figure 2. Pedigree of the kindred of proposita (arrow; II-7). Increased plasma HRG concentration is indicated by hatching and qualitatively abnormal HRG in black (Dot: not investigated and /: deceased).

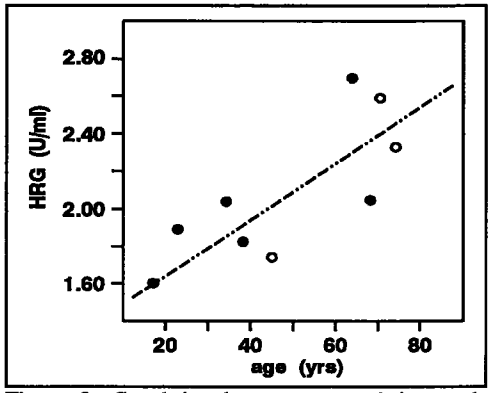

Figure 3. Correlation between age and increased plasma HRG level in proposita and eight relatives. Closed circles indicate subjects with qualitatively abnormal HRG and open circles those with normal HRG.

Table 2. Concentrations of plasma HRG in relatives of proposita.

Pedigree nr.	Age, sex	HRG (U/ml)	History of thrombosis
II-3	75, M	2.34	no
II-4	73, F	1.10	N.A.[+]
II-5	71, F	2.59	no
II-6	68, M	2.05	no
II-7[*]	64, F	2.70	yes
III-2	40, F	0.73	N.A.[+]
III-3	45, M	1.75	no
III-4	38, M	1.50	no
III-5	34, F	2.04	N.A.[+]
III-6	38, F	1.82	no
III-7	36, M	1.51	no
III-8	32, F	1.30	no
III-9	29, M	1.58	no
III-10	23, M	1.89	no
IV-1	15, M	1.52	no
IV-2	13, F	1.14	no
IV-3	17, M	1.60	no
IV-4	16, F	1.22	no

[*] proposita. [+] not available

Abnormal behaviour of HRG

Proposita's plasma HRG showed a slightly increased electrophoretic mobility in plain agarose; compared with normal pooled plasma the relative mobility was approximately 1.1 (range 1.07-1.13) (figure 4). When investigated in a crossed affinity immunoelectrophoresis system using heparin-containing gels, the mobility of proposita's HRG was evidently abnormal. The mobility of normal HRG in gels containing 100 U/mL standard heparin is clearly higher than in plain gels (figure 5a), but there was no such increase in mobility of the proposita's HRG (figure 5b). This difference remained present when EDTA was incorporated into the system for neutralizing the influence of divalent cations; single peaks were still observed. Also when proposita's plasma was diluted (with either immuno-depleted HRG-deficient plasma or saline) to give approximately 1.0 U/mL HRG, the abnormal heparin binding remained intact. Accordingly, we found this abnormal heparin-binding HRG in nine of 17 family

members; five of them had also increased HRG in their plasma (figure 2). The binding of HRG to low molecular weight heparin (LMWH; 100 IU/mL) was assessed in the same affinity electrophoresis system.
The mobility of normal HRG was essentially the same as in plain gels, which is much lower than in gels with standard heparin (figure 6a). The mobility of proposita's HRG in gels with LMWH was identical to that in plain gels and in gels with standard heparin (figure 6b). The binding of proposita's HRG to heparin-Sepharose was closely comparable to normal HRG; it eluted from the column at the same molarity (0.25 - 0.28 M NaCl) as HRG in normal pooled plasma (data not shown).

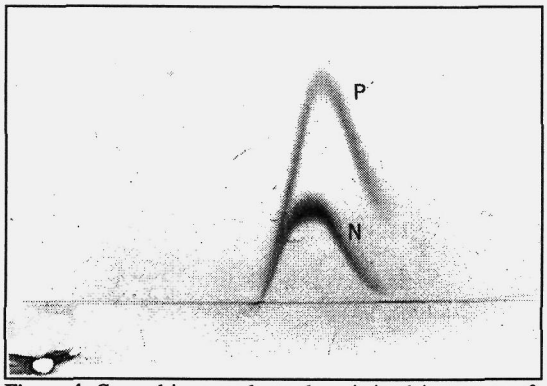

Figure 4. Crossed immunoelectrophoresis in plain agarose of normal pooled plasma (N) and proposita's plasma (P). Photograph of two overlayered gels. Electrophoresis in first dimension was with anode right and origin at bottom left.

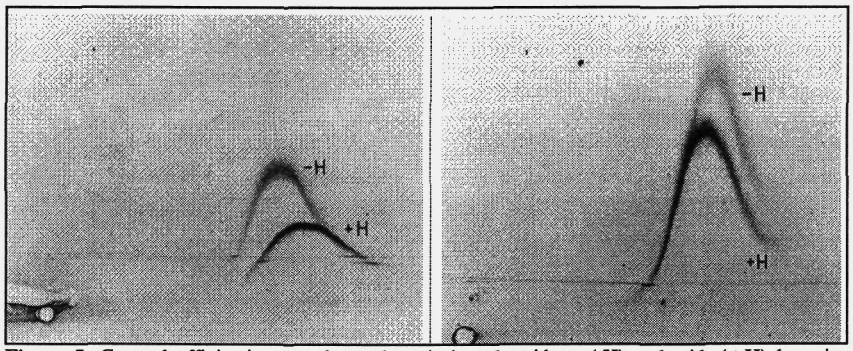

Figure 5. Crossed affinity immunoelectrophoresis in gels without (-H) and with (+H) heparin (100 U/mL). Normal plasma (**a**) and proposita's plasma (**b**).

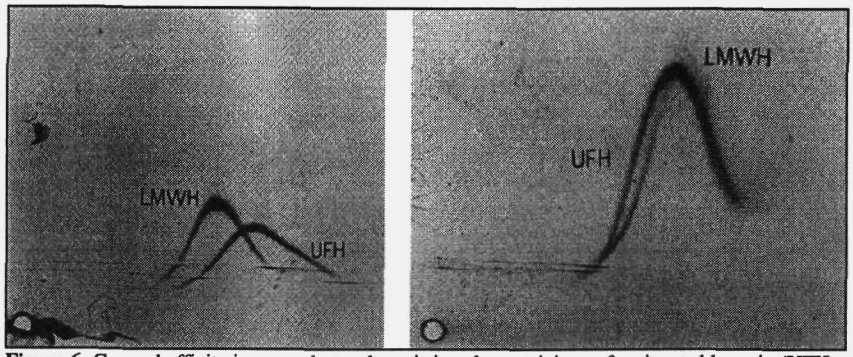

Figure 6. Crossed affinity immunoelectrophoresis in gels containing unfractionated heparin (UFH; 100 U/mL) and low molecular weight heparin (LMWH; 100 IU/mL). Normal plasma (**a**) and proposita's plasma (**b**).

The efect of heparin on the thrombin time of proposita's plasma as compared to normal plasma is illustrated in figure 7, showing that proposita's thrombin time was within the normal range. Also after dilution with immuno-depleted HRG-deficient plasma, to give approximately 1.0 U/mL HRG, the response to heparin remained within the normal range (figure 7).

Obviously, the concentration of HRG in platelets was too low to investigate whether proposita's platelet-HRG showed a similar behaviour as her plasma HRG. The binding of proposita's plasma HRG to plasminogen was normal, i.e. the relative mobility of her HRG-plasminogen complex was identical to that of normal plasma (not shown).

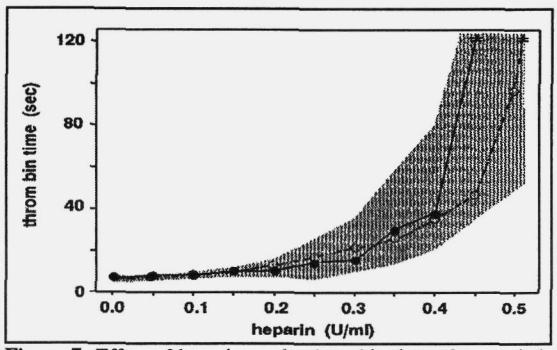

Figure 7. Effect of heparin on the thrombin time of proposita's plasma, undiluted (l) as well as diluted with HRG-depleted normal plasma to give 1.0 U/mL HRG (¡). Shaded area represents mean ± 2 SD range of 8 normal subjects.

Discussion

Although in selected patients with thrombophilia the incidence of increased HRG is 6-9%, which is clearly higher than in control subjects (Castel et al., 1983; Samama et al., 1983; Engesser et al., 1988), it still remains to be proven whether increased HRG is the cause of thrombosis. The association between increased HRG and thromboembolism is only partial and incomplete (Falkon et al., 1992; Anglés-Cano et al., 1993; Engesser et al., 1988; Shigekiyo et al., 1991). The available data are compatible with increased plasma HRG being hereditary and the family study of our proposita confirms these findings: figure 2 clearly suggests autosomal dominant inheritance of increased plasma HRG.
The discrimination between normal and increased plasma HRG concentration is not facile because of the non-Gaussian distribution (figure 1). Other authors have apparently neglected this problem in defining the upper limit of their normal range and therefore our value of 1.60 U/mL is slightly higher than in other studies (Castel et al., 1983, Samama et al., 1983; Engesser et al., 1988; Lijnen et al., 1981a). However, plasma HRG in our proposita and most of her family members was increased to such a degree that the increase is beyond any doubt. The HRG concentration of the proposita (between 2.55 and 2.90 U/mL) is indeed the highest ever published. Preliminary results from the ECAT angina pectoris trial indicate significant correlations between plasma HRG and body mass index and between plasma HRG and age; the latter correlation is mainly explained by post-menopausal women, who have the higher HRG (Thompson et al., 1991). Even after taking the obesity and age of our proposita into account, her plasma HRG remained extremely high.

Observations in the family described do not substantiate a possible causal association between increased HRG and thromboembolism. Of the 17 family members studied, only the proposita had a history of arterial thromboembolic events. None of the other relatives ever suffered from a documented thromboembolic disease. In addition to increased HRG, our proposita had undetectable tPA and increased PAI activity in her plasma (table 1), factors which are also known to be associated with recurrent thromboembolism (Hamsten et al., 1985; Nilsson et al., 1985; Paramo et al., 1985). This might indicate that increased HRG *per se* is not sufficient to cause thrombotic disease, but that only in combination with an additional factor, which further distorts the balance between coagulation and fibrinolysis, thromboembolism might develop without an apparent eliciting event. Recently, two other cases have been reported of familial increase in HRG with simultaneously increased PAI in patients with recurrent thrombosis (Falkon et al., 1992; Anglés-Cano et al., 1993); this supports the hypothesis that increased plasma HRG alone is probably no risk factor for developing thromboembolic disease.
Platelets are also a source of HRG (Leung et al., 1983), and therefore we determined platelet HRG in our proposita. Her platelets contained 590 ng/10^9 platelets, which is clearly higher than the reference range: 237-487 ng/10^9 platelets. Our reference mean

and range in 20 healthy subjects concur closely with the values found by Leung et al. (1983). So, platelet HRG of the proposita was approximately 160% of the normal mean. The abnormal binding of proposita's HRG to heparin was demonstrated using crossed affinity immunoelectrophoresis. Based on the pedigree (figure 2), our proposita is assumed to be heterozygous and therefore a pattern of two peaks, one normal and one abnormal, would have been expected. However, we found only a single peak of abnormally binding HRG, without a normal peak being detectable (figure 5). It is conceivable that the dominant peak of abnormal HRG (presumed to amount to 2.2 U/mL) concealed the much smaller peak of normal HRG (presumed to be approximately 0.5 U/mL), so that the latter escaped detection in the crossed affinity electrophoresis system. Alternatively, all HRG might be abnormal due to a dominant trait affecting protein processing. The exact nature of the abnormality must be elucidated in further studies before either of these possibilities can be rejected. In this respect it should be noted that ambiguity remains regarding the association of increased HRG and abnormal heparin binding, too. In six subjects high HRG and abnormal heparin binding was clearly associated, but in three others the increased concentration did not coincide with the heparin binding abnormality in the affinity electrophoresis system (figure 2); this might be due to the limited resolution of this system as well. In four individuals abnormal heparin-binding HRG was not associated with increased plasma concentrations according to our criteria. Yet we are confident that the heparin binding defect is not an artifact: it remained detectable after dilution to normal HRG concentration and, moreover, the pattern obtained after adding EDTA to the crossed affinity electrophoresis system, shows that the phenomenon is independent of divalent metal ions. It is necessary that the heredity of HRG is further investigated using molecular biology techniques for following the HRG allele in this family, in order to decide on the precise situation.

We have identified an intriguing syndrome of increased HRG associated with reduced heparin binding in crossed affinity immunoelectrophoresis. This technique suggests absence of high-affinity binding of heparin at 100 U/mL. The binding abnormality studied here is quite specific for unfractionated heparin, because LMWH did not show mobility changes with normal HRG (figure 6). This specificity appears to be uniquely expressed in the affinity electrophoresis system, since we did not observe differences in binding to heparin immobilized on sepharose. The heparin-neutralizing capacity of proposita's HRG in a clotting system was identical to that in normal plasma and therefore excludes a significant binding defect. It may be speculated that the heparin binding in the affinity electrophoresis system probed a molecular abnormality which is involved in abnormal regulation of the plasma concentration of HRG, either its synthesis or its clearance. It is anticipated that further analysis of this defect will provide us more insight in the regulation of plasma HRG and possibly in the relevance of heparin binding and the physiological role of HRG. We propose to name this first abnormal HRG variant after the city where it has been discovered: HRG Eindhoven.

Acknowledgements

The kind cooperation of the proposita and her family members is gratefully acknowledged. We thank Dr. J. Bonnier for his help, Mrs. P. Los for expert technical assistance and the physicians of the family members for providing information. B. Hennis was supported by grant 89004 from the Trombosestichting Nederland. This work was carried out within the framework of the European Concerted Action on Thrombosis and Disabilities (ECAT) of the Commission of the European Communities.

Chapter 4.2

Identification and genetic analysis of a common molecular variant of histidine-rich glycoprotein with a difference of 2 kDa in apparent molecular weight

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Submitted for publication.

Summary

Two forms of histidine-rich glycoprotein (HRG) were detected on SDS-PAGE by silver staining and immunoblotting after isolation of the protein from pooled plasma using immuno-affinity chromatography followed by chromatography with heparin-Sepharose. Both forms were single-chain molecules and the apparent molecular weights of form 1 and form 2 were 77 kDa and 75 kDa respectively. Mendelian inheritance of both HRG forms was observed in four families with 24 informative meioses strongly suggesting that the two forms are encoded by different alleles. The frequency of form 1 and form 2 in a group of 36 individuals was 0.35 and 0.65 respectively.

The difference between the two molecular variants was studied by direct sequence analysis of amplified exons of the HRG gene from 6 individuals who were homozygous either for form 1 or form 2. Five amino acid polymorphisms in three different exons were observed: Ile/Thr in exon 4; Pro/Ser in exon 5; His/Arg, Arg/Cys and Asn/Ile in exon 7. Analysis of these polymorphisms in 20 volunteers showed that only the Pro/Ser polymorphism at position 186 in exon 5 was coupled to the form of the HRG protein. Ser was found in form 1 and Pro in form 2. The presence of Ser at position 186 introduces a consensus sequence for a N-glycosylation site (Asn-X-Ser/Thr). A possible extra N-linked carbohydrate group at Asn 184 may therefore be the cause of the higher apparent molecular weight of form 1 of human HRG.

Introduction

Histidine-rich glycoprotein (HRG) is a single chain, non-enzymatic 3.8S a_2-glycoprotein that was first isolated from human plasma by Haupt et al. (1972). Besides its relatively high plasma concentration of 100 mg/ml (Heimburger et al., 1972) HRG is also stored in the a-granules of platelets (370 ng/10^9 platelets) from which it can be released upon thrombin stimulation (Leung et al., 1983). Plasma HRG is synthesized by the parenchymal cells of the liver (Hennis et al., 1991a) and the source of platelet HRG is most likely the megakaryocyte (Leung et al., 1983). In spite of all efforts to understand the significance of the many biological properties assigned to this multi-domain protein, the exact physiological function of HRG is still unknown. Due to interaction with heparin (Lijnen et al., 1983a), fibrinogen and fibrin (Leung et al. 1986), plasminogen (Lijnen et al., 1980) and activated platelets (Lerch et al., 1988), HRG is considered to be a modulator of coagulation and fibrinolysis (Koide, 1988b). Also a role in metal metabolism (Morgan, 1978b), and immunoregulation (Shatsky et al., 1989) among others has been described.

Proteins similar to HRG have been found in various species. HRG of ox (Muldbjerg et al., 1992), rabbit (Morgan, 1981), pig (Shimada et al., 1989) and mouse (Sia et al., 1982) has been isolated. Up to now only the cDNA sequence of human HRG has been elucidated. The cDNA sequence of HRG contains an open reading frame encoding a

protein of 507 amino acids of which 13% are histidines and an equal percentage are prolines (Koide et al., 1986a). The molecular weight including 14% carbohydrates is 66 kDa (Yip et al., 1991).
Purified human HRG subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) revealed a single band with an apparent molecular weight of about 75 kDa under reducing conditions (Lijnen et al., 1983a, Koide et al., 1985, Leung et al., 1989). No aberrant forms of HRG have been described yet. In this study however, we describe a molecular variant of HRG with a different molecular weight of 77 kDa on SDS-PAGE under reducing conditions.
In order to study a possible cause of this variation we analysed the coding sequence of the HRG gene in several individuals. Five different amino acid polymorphisms were found of which one is responsible for the difference observed on the protein level.

Materials and methods

Blood collection

This study was approved by the Medical Ethical Committee of HGO-TNO and informed consent was obtained from patients and volunteers.
Blood was collected from family members and volunteers by a single venipuncture into vacutainer tubes (Becton Dickinson, France) with sodium citrate as anticoagulant. Samples were kept on ice and centrifuged immediately at 3000g for 30 min at 4°C. Plasma was separated and stored at -80°C until use. The remaining blood cells and buffy coat of the samples were used for isolation of genomic DNA. Blood collected for large scale HRG isolations was drawn into tubes containing sodium citrate and 40 KIE/ml aprotinin (Trasylol; Pentapharm, Switzerland). Immediately after collection 1 mM diisopropylfluorphospate (DFP; Serva, Germany) was added.

Measurement of HRG

Concentrations of HRG were determined by an ELISA specific for HRG using rabbit anti-HRG antibodies raised against purified human HRG (Behringwerke, Germany). The procedure was as follows: microtitration plates (Flow Laboratories, U.K.) were coated overnight at 4°C with 150 ml rabbit-anti-HRG IgG (1.5 mg/well) in 0.05 M sodium carbonate buffer, pH 9.6. Prior to use, the wells were incubated with 10 mg/ml BSA (Sigma Chemical Co, Mo, USA) for 1.5 hour and subsequently washed three times with PBS-Tween (2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 145 mM NaCl, 1 mM Na_2EDTA, pH 7.4 and 0.1% Tween 20). Samples diluted in 100 ml PBS-Tween supplemented with 3% Polyethylene glycol 6000 (BDH chemicals, U.K.) were added and incubated for 2 hours at 37°C. After washing, the wells were incubated for 1.5 hours at room temperature with 100 ml horseradish peroxidase conjugated rabbit anti-HRG IgG properly diluted in sample buffer. Non-bound conjugate was washed away and 100 ml peroxide buffer containing the chromogenic substrate 3,3',5,5'-tetramethylbenzidine

(TMB; Organon Teknika, The Netherlands) was added. After 15 minutes at room temperature colour development was stopped with 100 ml 4M H_2SO_4 and the extinction was measured at 450 nm in a Titertek Multiscan spectrophotometer (Flow Laboratories, U.K.). Serial dilutions of citrated pooled plasma were used for calibration, assuming a HRG plasma concentration of 100 mg/ml (Lijnen et al., 1980). Control experiments using non-immune IgG coated plates showed no binding of HRG.

Purification of HRG

Two forms of HRG were isolated separately from plasma of homozygous individuals by immuno affinity chromatography followed by chromatography with heparin-Sepharose. Polyclonal antibodies from rabbit anti-HRG antiserum raised against human HRG (Behringwerke, Germany) were purified using protein A Sepharose 4B (Pharmacia, Sweden). These antibodies were coupled to CNBr activated Sepharose 4B (5 mg protein per ml gel) according to the manufacturers instructions (Pharmacia, Sweden). Freshly obtained citrated plasma containing 40 KIE/ml of aprotinin and 1 mM DFP was applied to an anti-HRG affinity column of 20 ml which was equilibrated with PBS (2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 145 mM NaCl, pH 7.5). The column was washed with 15 volumes of a PBS solution containing 1 M sodium chloride and subsequently HRG was eluted with 0.1 M glycine-HCl, pH 2.8. The eluate was immediately neutralized with 0.2 M Tris-HCl pH 8.5 and dialysed against 20 mM sodium phosphate, 0.4 M NaCl, pH 6.3. The dialysate was applied to a heparine-Sepharose column of 15 ml which was equilibrated with dialysis buffer. After washing the column with 5 volumes of 20 mM NaH_2PO_4, 0.8 M NaCl, pH 6.3, HRG was eluted with 20 mM NaH_2PO_4, 3 M NaCl, pH 8.0. The HRG concentration in the eluted fractions was determined with the HRG ELISA. Fractions containing HRG were pooled and dialysed against PBS. The entire procedure was carried out at 4°C.

Detection of HRG on SDS-PAGE

To separate the two forms 200 ng HRG was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) or to Schägger and von Jagow (1987) using 10% polyacrylamide gels. Before electrophoresis, samples were mixed with sample buffer with or without 2% b-mercaptoethanol as reducing agent and heated at 100°C for 3 min. HRG was either detected by silver staining according to Wray et al. (1981) or by immunoblotting.

The blotting procedure and subsequent immunostaining were performed as follows: after electrophoresis HRG was blotted onto a PVDF-membrane (Immobilon; Millipore, MA, USA) using a semi-dry blotting system (Pharmacia LKB Biotechnology, Sweden). Blotting was performed for 1.5 hours at 0.8 mA/cm^2 in dry-blot buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol). Following blotting, the membrane was incubated for 1 hour with 3% BSA in washing buffer (150 mM NaCl, 10 mM Tris, 0.05% Tween 20, pH 7.4). The membrane was rinsed with washing buffer and incubated for 1.5 hours with rabbit anti-HRG antibodies (Behringwerke, Germany).

Then, the membrane was incubated for 1.5 hours with horse radish peroxidase conjugated goat anti-rabbit antibodies (Nordic, The Netherlands) in washing buffer. Subsequently, the blot was stained by mixing 75 ml staining buffer (200 mM NaCl, 50 mM Tris, pH 7.4) with 15 ml methanol containing 45 mg 4-chloro-1-naphtol and 40 ml 30% peroxide solution. The staining reaction was stopped by rinsing the membrane with water.

Determination of HRG phenotypes

HRG phenotypes of individuals were determined using a small scale batch-wise isolation procedure. Sepharose coupled anti-HRG was incubated with 300 ml plasma overnight at 4°C in the presence of 40 KIE aprotinin/ml. After washing 3 times with 200 ml 10 mM sodium phosphate, 1 M NaCl, 40 KIE aprotinin/ml, pH 7.4, HRG was eluted with 0.1 M glycine-HCl, pH 2.8. Eluted fractions were neutralized with a half volume of 0.2 M Tris-HCl, 0.005% Tween-20, pH 8.5. HRG concentrations were determined using the HRG ELISA. 200 ng of this partly purified HRG was subjected to SDS-PAGE (Laemmli, 1970) and visualized by immunoblotting as described above.

Plasmin digestion of HRG

HRG was digested with plasmin to obtain fragments in order to determine the position of the 2 kDa difference in apparent molecular weight. 10 mg of HRG was incubated with 1 mg of plasmin in a volume of 100 ml for time periods varying from 30 seconds to 24 hours at 37°C. Plasmin activity was stopped by adding 1.0 mM D-Val-Phe-Lys-chloromethylketone (VFKCK; Calbiochem, CA, USA) followed by incubation at 37°C for 15 minutes. The degradation products were separated on SDS-PAGE (Laemmli, 1970) and visualized by silver staining according to Wray et al. (1981) or by blotting and subsequent immunostaining as described above.

Amino-terminal sequence analysis

100 pmol of various HRG samples were subjected to N-terminal sequencing on an Applied Biosystems Model 470A Protein sequencer, on-line equipped with a Model 120A PTH Analyzer.

In vitro amplification of exons and direct sequencing

Genomic DNA was obtained from freshly collected blood as described before by Wijmenga et al. (1990). Oligonucleotides were chosen complementary to intron sequences flanking the exons (table 1). Intron sequences were based on data of the complete nucleotide sequence of the HRG gene by Wakabayashi, S., Takahashi, K., Tokunaga, F. and Koide, T. (manuscript in preparation). In vitro amplification of each of the 7 exons was performed in a volume of 100 ml containing 0.5 mg genomic DNA; 200 ng of appropriate oligonucleotides of which one was 5'-biotinylated (Isogen Bioscience, The Netherlands); 200 mM dNTP; 1 x polymerase buffer and 0.1 unit Taq Polymerase (Amersham, U.K.). Reaction mixtures were overlaid with 2 drops of

mineral oil and placed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus, CT, USA). After a hot-start of 4 min at 94°C, thermocycling conditions were: 1 min at 94°C (denaturation), 1 min at 55°C (annealing), 2 min at 72°C (extension) for 30 cycles. Purification and direct sequencing of PCR products was performed according to a single strand sequencing method using magnetic beads coated with streptavidin (Menichini et al., 1991).

Table 1. Oligonucleotides used to amplify the different exons of HRG.

Exon	Oligonucleotides	Fragment length (bp)
1	1F1 5'-[b]TTCTCTGCAGTGGCAGATCATAGC-3' 1R1 5'-GTAATCTCCCAACTCAGCTCTGCC-3'	264
2	2F1 5'-[b]TTCCATGTGCTACTCACATGTTGC-3' 2R1 5'-AGAATGAAAAGAGCAGAGTGAAGG-3'	197
3	3F1 5'-AATGACTCAGCAAGTCCTCAAG-3' 3R1 5'-[b]GAATTAATTAGCAATTTAACAGATTACC-3'	161
4	4F1 5'-[b]TCCAGCCCTTTACTGTGACACTGC-3' 4R1 5'-TGGGCCTTCAGACTCCAACCGAAC-3'	247
5	5F1 5'-[b]CTGTTCTTGAAACTATTTGATCC-3' 5R1 5'-TGACTCTAGTCAACGATCAC-3'	156
6	6F1 5'-[b]ACCTGGACACACACACTAACAGCT-3' 6R1 5'-CGTATGTCACTTAATCTGCACTGC-3'	194
7	7F1 5'-[b]GATGATAGGCACTTTTCTGTGACC-3' H2B 5'-TATTATTCATTTTCCTCTTCAAAGG-3'	903

[b] 5'-biotinylation of the oligonucleotide.

Analysis of polymorphisms

All polymorphisms were detected by restriction enzyme analysis of in vitro amplified fragments. The alleles of the polymorphism differed from each other by one restriction site. Restriction enzymes used: Hinf I (Amersham, U.K.); Bpm I, Alw NI, Xmn I (New England Biolabs, MA, USA); Mae II (Boehringer Mannheim, Germany). 5 ml of PCR product was digested with the appropriate restriction enzyme in a total volume of 10 ml according to the instructions of the manufacturer. Restriction patterns were analysed on agarose gels containing ethidium bromide.

Results

Two molecular weight forms of HRG

A double band was observed after isolation of HRG from pooled plasma followed by SDS-PAGE and immunoblotting indicating the presence of two forms of HRG (fig. 1). The frequencies of the two forms were determined in 36 healthy volunteers. HRG was isolated from plasma using a small scale batch-wise isolation procedure with anti-HRG-Sepharose. A typical yield of 1-3 mg HRG was obtained from each individual. The HRG phenotypes were determined by SDS-PAGE followed by immunoblotting. Both single and double bands were observed in several individuals. 6 individuals had only form 1, 17 only form 2 and 13 had both form 1 and 2. The calculated frequencies for form 1 and form 2 in this group were 0.35 and 0.65 respectively.

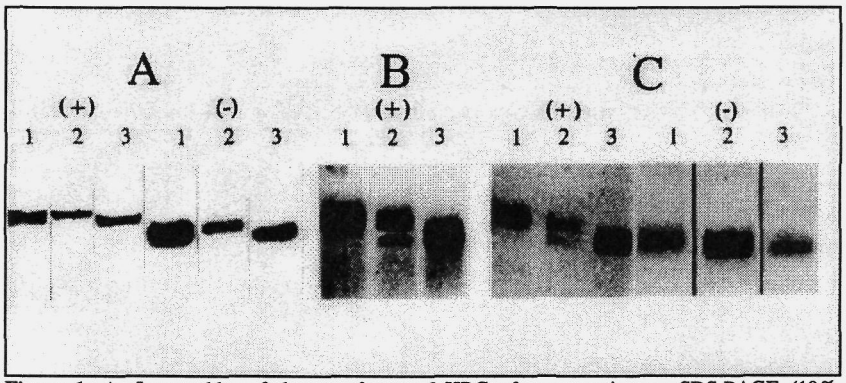

Figure 1. A. Immunoblot of the two forms of HRG after separation on SDS-PAGE (10% acrylamide) utilizing a tris-glycine buffer according to Laemmli (1970). Lane 1: mixture of form 1 and 2, lane 2: form 1 (77 kDa), lane 3: form 2 (75 kDa). B. Immunoblot of the two forms after separation on SDS-PAGE (10% acrylamide) using a tris-tricine buffer (Schägger and von Jagow, 1987). Lane 1: form 1 (77 kDa), lane 2: mixture of form 1 and 2, lane 3: form 2 (75 kDa). C. Silver staining of gel identical to B. Lane 1: form 1 (77 kDa), lane 2: mixture of form 1 and 2, lane 3: form 2 (75 kDa). (+) : after reduction with 2% b-mercaptoethanol, (-) : non-reducing conditions.

Heredity

Mendelian inheritance of both forms was observed in 4 families with 24 informative meiosis. In figure 2 a part of a family is shown with the inheritance pattern of the two forms. This inheritance pattern strongly suggests that the two protein variants of HRG are encoded by two different alleles of the HRG locus.

Mobility of HRG forms on SDS-PAGE

The two variants were isolated separately using plasma of individuals apparently homozygous for form 1 or form 2. Homogeneity of the protein preparations was examined by SDS-PAGE followed by silver staining. Possible proteolytic degradation arising from the isolation procedure was checked by SDS-PAGE followed by

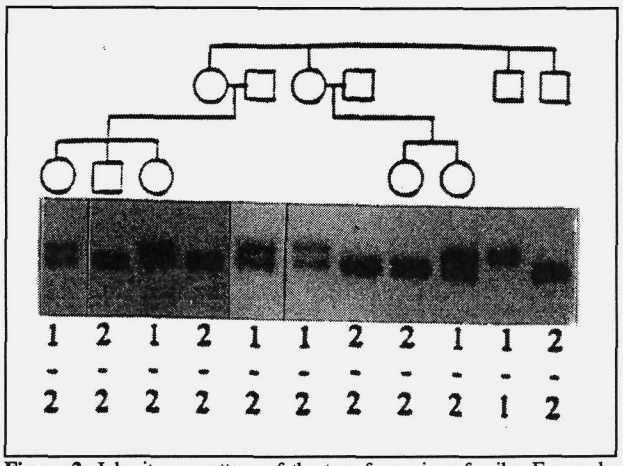

Figure 2. Inheritance pattern of the two forms in a family. For each individual 200 ng of HRG was used. The two forms were separated on SDS-PAGE (Laemmli, 1970) and visualized by immunoblotting. Phenotypes of family members are noted at the bottom.

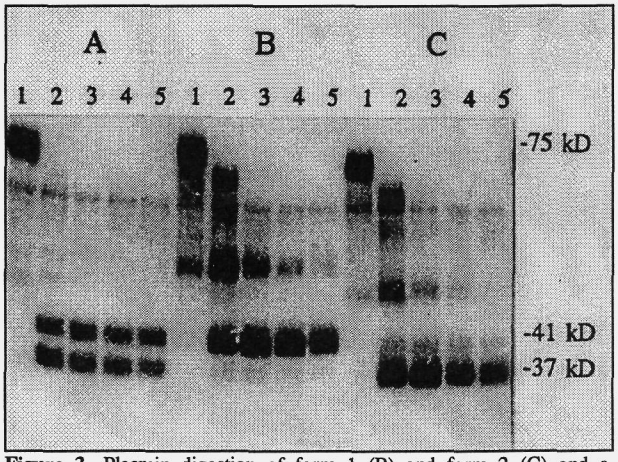

Figure 3. Plasmin digestion of form 1 (B) and form 2 (C) and a mixture of both forms (A). Per lane 500 ng HRG was used. Degradation products were separated on SDS-PAGE (Laemmli, 1970) and visualized by silver staining. Digestion was performed with 10 mg HRG and 1 mg plasmin in a volume of 100 ml for time periods of 30 seconds (lanes 2), 2 hours (lanes 3), 6 hours (lanes 4) and 24 hours (lanes 5). Lanes 1 contain native HRG.

immunoblotting. Both forms were obtained in quantities of 2-3 mg (typical yield of 60-70%) and highly purified (>95%). A difference in migration velocity of the two forms was observed both under reducing and non-reducing conditions on SDS-PAGE (10% acrylamide) utilizing either a tris-glycine (Laemmli, 1970) or a tris-tricine (Schägger and

von Jagow, 1987) buffered system (figure 1). In both systems the difference in apparent molecular weight between the two forms was about 2 kDa.

Proteolytic digestion and amino-terminal analysis

The location of the difference in apparent molecular weight between both HRG forms was studied by proteolytic degradation using plasmin (figure 3). After complete digestion (24 hrs) the difference appeared to be located in a fragment of about 40 kDa which was previously identified as the N-terminus of HRG (results not shown). This fragment remained resistant to further proteolytic degradation by plasmin.

From each native HRG form, 42 residues of the amino-termini were sequenced. All amino acids of form 1 and form 2 were identical to the previously reported amino acid sequence as deduced from the cDNA sequence (Koide et al., 1986a) (results not shown).

Exon sequencing

Genomic DNA was isolated from 3 individuals homozygous for form 1 and from 3 individuals homozygous for form 2. All 7 exons were in vitro amplified and nucleotide sequences were determined subsequently. Comparison of these exon sequences with the previously reported HRG cDNA sequence (Koide et al., 1986a) showed 5 single-base pair mutations all leading to amino acid substitutions (table 2). In addition, one neutral base pair mutation of C to T was found at position 238 in exon 1 of all individuals analysed indicating a rare mutation or a sequence artefact in the published cDNA sequence (Koide et al., 1986a).

Table 2. Base pair substitutions (in capitals) and the deduced amino acid substitutions observed in 6 individuals (3 homozygous for form 1 and 3 homozygous for form 2).

Exon	Position of base pair	Base pair substitution	Position of amino acid	Amino acid substitution
4	660	aTc ® aCc	162	Ile ® Thr
5	731	Ccc ® Tcc	186	Pro ® Ser
7-1	1140	cAt ® cGt	322	His ® Arg
7-2	1464	Cgt ® Tgt	430	Arg ® Cys
7-3	1599	aAt ® aTt	475	Asn ® Ile

Base pair substitutions are shown in capitals. Positions of base pairs and amino acids are according to the cDNA sequence reported by Koide et al. (1986a).

HRG genotypes in 20 volunteers

Genotypes for the polymorphisms in exon 4, exon 5 and exon 7 (7-1, 7-2 and 7-3) were determined by in vitro amplification of a fragment containing the polymorphism followed by restriction enzyme analysis. Oligonucleotides used for amplification, the length of the PCR products and the restriction fragments obtained are shown in table 3. In case of polymorphism 7-2 and 7-3 no restriction site was found which included the polymorphic

base pair. For these polymorphisms oligonucleotides containing mismatches were designed to introduce restriction sites that could discriminate between the two alleles of each polymorphism (table 3) (Russ et al., 1993). An Alw NI restriction site was introduced for polymorphism 7-2 whereas for 7-3 a restriction site for Xmn I was introduced.

The genotypes with respect to the amino acid polymorphisms were determined in 20 unrelated volunteers (table 4). Both alleles of each polymorphism were present in this group and calculated allele frequencies were in the range of 12% - 88% (table 5).

Table 3. Oligonucleotides used to amplify fragments containing polymorphisms.

Polymorphism in:	Oligonucleotides	Restriction enzyme	Restriction fragments (bp)
Exon 4	4F1 5'-[b]TCCAGCCCTTTACTGTGACACTGC-3' 4R1 5'-TGGGCCTTCAGACTCCAACCGAAC-3'	Hinf I	Ile: 6,179,25,23,14 Thr: 6,204,23,14
Exon 5	5F1 5'-[b]CTGTTCTTGAAACTATTTGATCC-3' 5R1 5'-TGACTCTAGTCAACGATCAC-3'	Bpm I	Pro: 156 Ser: 84,72
Exon 7-1	7F1 5'-[b]GATGATAGGCACTTTTCTGTGACC-3' H2B 5'-TATTATTCATTTTCCTCTTCAAAGG-3'	Mae II	His: 903 Arg: 310,593
Exon 7-2	7F2 5'-AGGCGAGGCCCAGGTAAcaGACCC-3' E9R 5'- TTATTTTGGAAATGTATGTGTAAAAAACATGG-3'	Alw NI	Arg: 178,76 Cys: 162,76,16
Exon 7-3	7F3 5'-ACCACAAACATCCTCTAAAGgaAGACA-3' E9R 5'- TTATTTTGGAAATGTATGTGTAAAAAACATGG-3'	Xmn I	Asn: 129 Ile: 104,25

Mismatches in oligonucleotide 7F2 and 7F3 are shown in lower case. Restriction enzymes used to detect the polymorphisms and the restriction fragments obtained are shown. [b] 5'-biotinylation of the oligonucleotide.

Relationship between HRG genotype and phenotype

The genotype of each individual was compared to its accompanying phenotype (table 4). The only genotypic polymorphism related to the phenotype of the HRG protein is located in exon 5. Homozygous individuals for form 1 are also homozygous for Ser at position 186 in exon 5 whereas homozygosity for form 2 is related to homozygosity for Pro at this position. The genotype with respect to this Pro/Ser polymorphism is found in combination with various genotypes of the other polymorphisms. However, the other polymorphisms did not show any noticeable influence on the phenotype of HRG.

Table 4. The relationship between the HRG phenotype and the genotype with respect to the 5 amino acid polymorphisms in 20 volunteers.

Ind.	Form of HRG	Exon 4 Ile - Thr +	Exon 5 Pro - Ser +	Exon 7-1 His - Arg +	Exon 7-2 Arg - Cys +	Exon 7-3 Asn - Ile +
1	1-2	-	+/-	-	-	+/-
2	1-2	-	+/-	-	-	+/-
3	2-2	-	-	-	+/-	+/-
4	1-2	-	+/-	+/-	+/-	+
5	1-1	-	+	+/-	-	+
6	1-2	-	+/-	-	-	+/-
7	1-2	+/-	+/-	-	+/-	+/-
8	1-2	+/-	+/-	-	+	+
9	1-1	+/-	+	+/-	-	+
10	2-2	-	-	-	-	-
11	2-2	-	-	-	-	-
12	2-2	-	-	-	-	-
13	1-2	-	+/-	+/-	+/-	+
14	2-2	-	-	-	-	-
15	1-1	+/-	+	+/-	+/-	+
16	1-2	+/-	+/-	-	+/-	+/-
17	2-2	-	-	-	-	-
18	2-2	-	-	-	+/-	+/-
19	1-2	-	+/-	+/-	-	+/-
20	1-2	-	+/-	+/-	-	+/-

Genotypes were determined by restriction enzyme analysis of amplified exons. Amino acids were deduced from the genotypes. - = common allele and + = more rare allele.

Table 5. Allele frequencies of the polymorphic amino acids in HRG calculated from 20 volunteers

Exon	Genotype: No. of individuals			Allele frequency	
4	Ile -	Ile	:15	Ile	: 0.88
	Ile -	Thr	: 5	Thr	: 0.12
	Thr -	Thr	: 0		
5	Pro -	Pro	: 7	Pro	: 0.6
	Ser -	Pro	: 10	Ser	: 0.4
	Ser -	Ser	: 3		
7-1	His -	His	: 13	His	: 0.82
	Arg -	His	: 7	Arg	: 0.18
	Arg -	Arg	: 0		
7-2	Arg -	Arg	: 12	Arg	: 0.77
	Cys -	Arg	: 7	Cys	: 0.23
	Cys -	Cys	: 1		
7-3	Asn -	Asn	: 6	Asn	: 0.53
	Asn -	Ile	: 9	Ile	: 0.47
	Ile -	Ile	: 5		

Amino acids were deduced from the genotype which was determined using restriction enzyme analysis.

Discussion

Since the discovery of histidine-rich glycoprotein in 1972 several different isolation procedures have been published in which histidine-rich glycoprotein was isolated from plasma (Lijnen et al., 1983a; Leung et al., 1989; Koide et al., 1985). Up to now plasma HRG has been reported as a single band on SDS-PAGE both under reducing and non-reducing conditions. However, the present study showed that during the isolation of HRG from pooled plasma two forms of HRG were isolated. The two molecular variants appeared as a doublet on SDS-PAGE with an apparent molecular weight of about 77 kDa and 75 kDa (form 1 and form 2, respectively) both under reducing and non-reducing conditions. Both forms followed a Mendelian inheritance pattern in 4 families indicating that the two forms are encoded by two different alleles. With respect to the allele frequencies of 0.35 and 0.65 (for form 1 and 2 respectively) in 36 unrelated volunteers the two forms have to be considered as a polymorphism (Peake et al., 1991).
By proteolytic fragmentation of both forms with plasmin, the difference in molecular weight could be localized in a fragment of about 40 kDa which represents the N-terminal part of HRG. This fragment is about the size of the two cystatin domains of HRG (amino acid 1-229) which are encoded by the first six exons of the HRG gene (Koide, 1988a). The resistance to further proteolytic degradation of this fragment may indicate a compact structure of the cystatin domains. This is also suggested by the presence of five

disulphide-bridges in these domains which has been proposed by Sørensen et al. (1993) in analogy to bovine HRG.

As a strategy to identify the difference between the two forms, we analysed the coding sequence of the HRG gene of individuals homozygous for form 1 or form 2. Five polymorphisms resulting in single amino acid substitutions were discovered by direct sequencing of all exons in 6 individuals. The polymorphism in exon 4 at position 162 may either be an Ile or a Thr. Although the presence of a Thr may lead to the introduction of an O-linked carbohydrate, this polymorphism is not related to one of the forms of HRG on the protein level. A second polymorphism, in exon 5 at position 186 is formed by a Pro and a Ser. This polymorphism is obviously related to the phenotype of HRG: in form 1 amino acid 186 is exclusively Ser whereas in form 2 it is Pro (table 4). It is noteworthy that the two preceding amino acids are an Asn and a Cys, and since the consensus sequence for a N-linked glycosylation site is Asn-X-Ser/Thr, the substitution of Pro by Ser introduces a possible extra N-linked glycosylation site at Asn-184 in form 1.

Three additional polymorphisms were found in exon 7 at positions 322 (7-1), 430 (7-2) and 475 (7-3). None of these polymorphisms is coupled to either form of HRG. A His/Arg polymorphism is located at position 322 between the first proline-rich region (aa 255-314) and the histidine-rich region (aa 330-389) (Koide, 1988a). The amino acid at position 430 is either an Arg or a Cys. The introduction of a half-cystine may be interesting, but as yet we do not know whether this half-cystine is interfering with the disulfide-bridge formation of the other 16 half-cystines of the molecule. The polymorphism at position 475 (Asn/Ile) is located in the unique C-terminal part of HRG and has also been identified by peptide sequencing (T. Koide, unpublished result). The amino acid residue at this position in bovine HRG is an Ile.

From our study it can be concluded that the Pro/Ser polymorphism in exon 5 is responsible for the variation in molecular weight and it is interesting to speculate on the cause of this difference. Unfortunately, little is known about the secondary and tertiary structure of human HRG. The only knowledge of its structure has been derived from the homology of HRG to members of the cystatin super family (Koide et al., 1987). The recent elucidation of the partial amino acid sequence and the disulphide bridge arrangement of bovine HRG (Sørensen et al., 1993) provides additional information although the amino acid homology is relatively low (65%) and only 12 half-cystine residues have been found in bovine HRG compared to 16 in human HRG.

The presence of a N-linked carbohydrate group at Asn-184 in bovine HRG is interesting. This N-glycosylation site is non-heterogeneous and the consensus sequence is formed by exactly the same amino acids (Asn-Cys-Ser) as found in form 1 of human HRG. Analogous to bovine HRG the Asn-184 in form 1 may be glycosylated. It is therefore possible that the higher apparent molecular weight of form 1 of human HRG is caused by a carbohydrate-dependent mobility change. However, it has been reported that the mutational substitution of a single amino acid can affect the mobility of a protein in SDS-PAGE (Noel et al., 1979). Consequently, it is still possible that the heterogeneity in

mobility has to be ascribed to structural changes due to the Pro/Ser polymorphism. Intended analysis of the carbohydrate content of both forms will provide more information.
The observed heterogeneity in the HRG molecule may have consequences for the physiological role of HRG in thrombosis. In recent publications several thrombophilic families with a familial elevation of HRG levels have been described (Castaman et al., (1993), Anglés-Cano et al., (1993), Engesser et al., (1987)). However, up to now no causal relationship between the elevated HRG levels and thrombosis could be found. It is therefore tempting to speculate on a role of specific combinations of the above described polymorphisms in these families. In this respect it may be interesting to determine haplotypes in normal and thrombophilic families.

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Chapter 5

HRG alleles and plasma HRG levels

Chapter 5.1

A specific allele of the histidine-rich glycoprotein (HRG) locus is linked with elevated plasma levels of HRG in a Dutch family with thrombosis

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Summary

Recent studies describe families with both elevated plasma HRG levels and thrombosis. In order to study the possibility that allelic variants of the HRG locus are associated with differences in HRG level, we studied linkage between HRG levels and a dinucleotide repeat polymorphism in a Dutch family which was selected on the presence of both thrombosis and elevated plasma HRG levels. No other known risk factors for thrombosis were found in this family. Linkage was calculated between the dinucleotide repeat and the HRG level considering the HRG level as a quantitative phenotype assuming a population prevalence of elevated HRG of 5%. Two classes of HRG levels were defined by a mean and a variance: one class with normal HRG levels and a second class with high HRG levels. Using a mean HRG level of 99% for individuals with a normal HRG level and 145% for individuals with high HRG, a maximum lod score of 4.17 (odds in favour of linkage of 22,000:1) was found at a recombination fraction of 0, indicating linkage. Considering the pedigree, an association was found between the presence of a specific allele (no. 6) of the dinucleotide repeat polymorphism and plasma HRG levels. Family members carrying allele 6 were found to have higher HRG plasma levels compared with family members lacking allele 6 (149% versus 109% respectively). We conclude that in this family linkage is found between the HRG locus and the HRG level and that a HRG gene coupled to allele 6 of the dinucleotide polymorphism is associated with elevated plasma HRG levels. No evidence was found for a causal relationship between elevated plasma HRG levels and thrombosis in this family.

Introduction

Histidine-rich glycoprotein (HRG) is a single-chain glycoprotein which is found in human plasma at a concentration of 100 mg/ml (Heimburger et al., 1972). A pool of HRG is also stored in the a-granules of platelets at a concentration of about 0.4 mg/10^9 platelets (Leung et al., 1983; Hoffmann et al., 1993). Plasma HRG is produced by the parenchymal cells of the liver (Hennis et al., 1991) and the source of platelet HRG is probably the megakaryocyte (Leung et al., 1983). Up to now the exact physiological function of HRG is unknown but it has been proposed that HRG can act as a modulator of coagulation and fibrinolysis (Koide, 1988). HRG has been shown to interact with a variety of haemostatic factors like heparin (Koide et al., 1982), fibrinogen (Leung et al., 1986) and plasminogen (Lijnen et al., 1980). The ability of HRG to bind plasminogen is thought to affect fibrinolysis by reducing the amount of fibrin-bound plasminogen which is available for activation into plasmin. An elevation of the HRG level in plasma may therefore reduce the fibrinolytic potency.
Supporting evidence for this hypothesis is presented in several studies of patients with thrombosis (Samama et al., 1983; Engesser et al., 1988; Ehrenforth et al., 1994). In

these patients a higher prevalence of elevated HRG levels has been observed. Moreover, in recent years six families have been described in which both thromboembolic disease and elevation of plasma HRG was observed (Falkon et al., 1991; Anglés-Cano et al., 1993; Castaman et al., 1993; Hoffmann et al., 1993; Engesser et al., 1987). All members of these families with a history of thrombosis had elevated levels of HRG although also a few family members had elevated HRG levels and no history of thrombosis. However, up to now it is unknown whether the relationship between congenitally elevated HRG levels and thrombosis is causal or a coincidental.
Recently, a twin study was presented in which genetic factors were found to explain 69% of the variance in plasma HRG levels (Boomsma et al., 1993). From this study it is clear that a familial elevation of HRG levels cannot be attributed to common environmental influences shared by family members, but has to be attributed to genetic influences shared by parents and children. Several genes, among them the HRG gene which is localized on chromosome 3q28-q29 (Hennis et al., 1994), may be responsible for this genetic influence. In some reports it has been speculated that the inherited elevated HRG levels find their cause in an abnormal HRG gene (Hoffmann et al., 1993; Schved et al., 1991), but up to now no association has been reported between elevated HRG levels and a specific allele of the HRG gene.
To investigate the role of the HRG locus in congenital elevation of HRG, we studied the inheritance pattern of a multi-allelic dinucleotide repeat polymorphism within the HRG gene (Hennis et al., 1994) in a Dutch family with both elevated HRG levels and thrombosis. Quantitative linkage analysis suggested involvement of the HRG locus. One allele of the highly informative dinucleotide repeat was found to be linked with high plasma HRG levels.

Methods

Case report and family history

The proband with thromboembolic disease and elevated HRG came to our attention in a previous study (Engesser et al., 1988). The proband (II-18, see pedigree in figure 1) was a 46 year old man seen for episodes of recurrent thromboembolism; deep venous thrombosis (DVT) in the right leg and pulmonary embolism after groin rupture surgery at the age of 32; one year later DVT in the left leg after stopping oral anticoagulant therapy with coumarin. At the age of 35 a spontaneous thrombosis was diagnosed in the right leg. In all three episodes DVT was confirmed by phlebography.
The pedigree is shown in figure 1. Persons with a history of thrombosis are indicated. Both parents of the patient suffered from DVT. The father (I-1) had DVT in the right leg at the age of 77. The mother (I-2) had DVT in the right leg at the age of 75. One of the proband's sisters (II-1) had recurrent DVT after an operation at the age of 40. A brother of the proband (II-3) had DVT in the right leg at the age of 29 after a

trauma. One family member (II-14) that is related by marriage had postoperative DVT in a leg. Another family member (II-12) that is related by marriage was treated with percutane transluminal coronairy angiography (PTCA).
Oral contraceptives were used by four females in generation III (11, 15, 17 and 18).

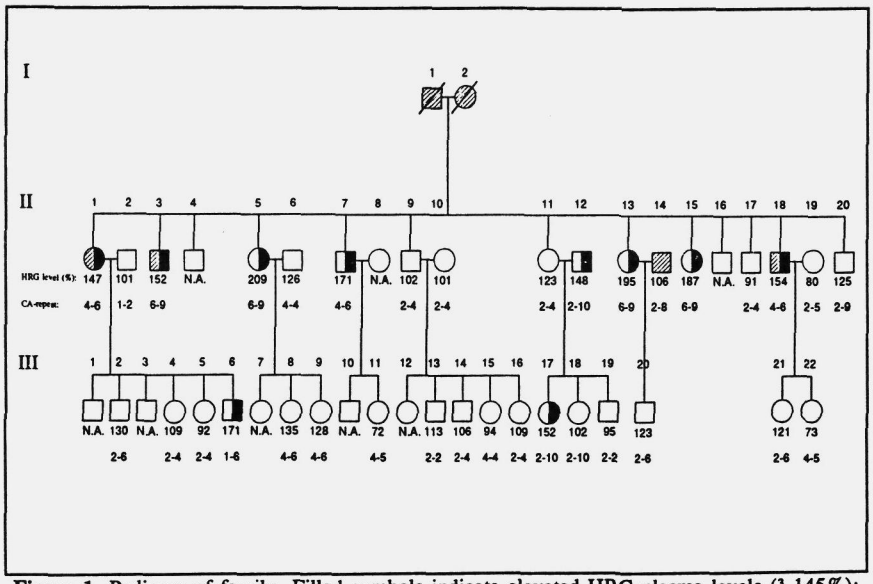

Figure 1. Pedigree of family. Filled symbols indicate elevated HRG plasma levels (^{3} 145%); Dashed symbols indicate a history of thrombosis. Family members that were not available for investigation are indicated with N.A.. The HRG level and the genotype with respect to the dinucleotide repeat and the Kpn I RFLP are shown below each individual. The proband is indicated by an arrow.

Blood collection

This study was approved by the Medical Ethical Committee of HGO-TNO and informed consent was obtained from patients and controls.
Blood was collected from family members by a single venipuncture into vacutainer tubes (Becton Dickinson, France) with sodium citrate as anticoagulant. Samples were kept on ice and centrifuged immediately at 3000g for 30 min at 4°C. Plasma was separated and stored at -80°C until use. The remaining blood cells and buffy coat of the samples were used for isolation of genomic DNA.

Coagulation assays

Assays of activated partial thromboplastin time, prothrombin time, thrombin clotting time, fibrinogen (Clauss method) and factor VIII coagulant activity were performed according to established procedures. Coagulant activities of factor V and VII were measured by one-stage clotting assays, using factor V and VII deficient plasma and rabbit brain thromboplastin (Simplastin-plus, General Diagnostics, Morris Plains,

USA). Protein S antigen was measured by a radioimmunoassay previously described by Bertina et al., (1985). Antithrombin III activity was determined by a spectrophotometric assay (Coatest, Kabivitrum, Sweden). Von Willebrand factor antigen, factor II and factor X antigen, heparin cofactor antigen (Bertina et al., 1987) and protein C antigen (Bertina et al., 1982) were measured by Laurell rocket electroimmunoassay. The anticoagulant response to activated protein C (APC) was measured using Coatest® APC resistance (Chromogenix, Sweden). Protein C activity was determined using a new assay based on the measurement of coagulation times on a Coag-A-Mate® RA-4 (Organon Teknika, The Netherlands). The assay has been developed by Organon Teknika and will be available in the near future. The procedure was started by the reconstitution of a vial of Protac (Pentapharm, Switzerland) in 6 ml APTT-L (Platelin-L®, Organon Teknika) reagent (Protac concentration: 0.5U/ml). The APTT/Protac solution was allowed to stand for 15 min. at 0°C. A calibration curve was made by diluting Verify® Reference Plasma (Organon Teknika) 0, 5 and 10 times in Protein C depleted plasma. Plasma of patients was diluted 10 times in Protein C depleted plasma. Cuvets containing 100 ml of the calibration solutions or the diluted samples were inserted into the RA-4 and the measurement was started. After a fixed heating time, which cannot be influenced, 125 ml of APTT/Protac solution was added via the Coag-A-Mate® RA-4. Following an incubation time of 220 seconds at 37°C, 100 ml $CaCl_2$ solution (Platelin®, Organon Teknika) was added and coagulation times were registered. Protein S activity was determined with essentially the same procedure using however protein S deficient plasma, 125 ml $CaCl_2$ and 100 ml of APTT/Protac.

Fibrinolysis assays

Plasminogen (Plg) activity was determined on a MLA 1000C (Medical Laboratory Automation, Pleasantville, New York, USA) using a chromogenic assay (Dade®, Baxter, Miami, USA). For calibration Dade® CoagCal®N (Lot. no. 540.046, Baxter, Miami, USA) was used. The assigned value of the plasminogen level in this plasma is 106%. a_2-antiplasmin activity was measured by a chromogenic assay according to Friberger et al. (1978). Tissue-type plasminogen activator (t-PA) activity was measured according to Verheijen et al. (1982). Urokinase-type plasminogen activator (u-PA) antigen and activity were determined using an ELISA (Binnema et al., 1986) and a biological immunoassay (Van Hinsbergh et al., 1990), respectively. Fibrinolytic activity of the intrinsic plasminogen activating system was determined on fibrin plates, using euglobulin fractions from plasma, prepared in the presence of dextran sulphate, with or without the addition of antibodies against u-PA (Kluft et al., 1984). Plasminogen activator inhibitor (PAI) activity was measured according to Verheijen et al. (1984).

Assay of histidine-rich glycoprotein

Measurement of plasma HRG levels was performed by radial immunodiffusion (Mancini et al., 1965) as previously described (Boomsma et al., 1993). Using this

method HRG levels were determined in 126 healthy individuals. The mean HRG level was 99% (SD=20%) and the range was 56%-145%.
Electrophoretic mobility of HRG in the presence of Glu-plasminogen was studied by affinity crossed immunoelectrophoresis according to Kluft et al. (1988). Heparin binding was studied by affinity crossed immunoelectrophoresis with 100 IU heparin per ml (Thromboliquine, Organon Teknika, The Netherlands) and 1 mM $CuCl_2$ in the first dimension (Hoffmann et al., 1993).

Detection of polymorphisms
Genomic DNA was obtained from freshly collected blood as described previously (Wijmenga et al., 1990). Genotypes with respect to the dinucleotide repeat were detected by use of the polymerase chain reaction (PCR) with radio-labelled nucleotides (Hennis et al., 1992), using primers HRG-CA2F: 5'AAG CAG ACT TTG TCA TGG CAG TGC 3' and HRG-CA2R: 5' TTG CAC TCC TTT CCC CAG TTG TGG 3'(Isogen Bioscience, Amsterdam, The Netherlands). Individual genotypes were determined using a standard set of repeats of known length (figure 2). The repeat is localized in the last intron of the HRG gene (Hennis et al.,1994).

Linkage
Linkage was carried out using the MLINK option of the LINKAGE computer program package, version 5.03 (Lathrop et al., 1988). Linkage was calculated between the phenotype 'HRG level' and the dinucleotide repeat that is located in the last intron of the HRG gene. HRG levels were considered as a quantitative phenotype under the assumption that elevated HRG is a monogenic dominantly inherited trait (Ott, 1991). Two classes were distinguished: one with normal HRG levels (unaffected) and a second with high HRG levels (affected). In this approach the two classes were defined by a mean HRG level and a variance. For the class with normal HRG levels, a mean HRG of 99% and a variance of 400 was used in all calculations. This mean level and variance were derived from a control group of 126 volunteers. For the class with high HRG levels lod scores were calculated for mean levels of 135%, 145%, 155% and 165%. The variance of HRG levels was assumed to be equal for both classes. The population frequency of high HRG levels was assumed to be 0.05. No corrections for possible age effects on the HRG level have been implemented in the linkage analysis.

Results

Plasma levels
In order to exclude the possibility that the apparent familial thrombophilia was due to known or suspected disorders of fibrin formation and fibrinolysis, we studied the proband extensively on coagulation and fibrinolysis parameters (table 1).

Table 1. Coagulation and fibrinolysis parameters in the proband's plasma.

	Proband*	Normal range#
Clotting times		
APTT (s)	29.4	28.0-39.2†
Prothrombin time (s)	17.0	17.0-19-0†
Thrombin clotting time (s)	16.1	14.3-18.5†
Coagulation factors		
Fibrinogen (mg/ml)	2.2	1.8-4.9
Factor II antigen (%)	57	46-77†
Factor V activity (%)	100	52-124
Factor VII activity (%)	28	60-157
Factor VIII coagulant activity (%)	172	50-200
Factor X antigen (%)	61	31-76†
Von Willebrand factor antigen (%)	165	31-216
Antithrombin III activity (%)	91	75-120
Heparin cofactor II antigen (%)	115	61-185
Protein C antigen (%)	55	40-70†
Protein S antigen (%)	60	36-66†
Fibrinolysis factors		
Plasminogen activity (%)	113	70-140
t-PA activity (mIU/ml)	13.2	> 0
u-PA activity (ng/ml)	3.5	1.3-3.1
u-PA antigen (ng/ml)	4.3	1.7-6.2
Factor XII-dependent plasminogen activator activity (%)	95	76-158
Plasminogen activator inhibitor activity (IU/ml)	6.4	1.7-34.0
a_2-antiplasmin activity (%)	95	80-120
HRG antigen (%)	161/154‡	56-145

* Oral anticoagulant treatment at the time of investigation with an intensity of 2.07 International Normalized Ratio. # Normal ranges were established in at least 42 healthy members of the laboratory staff. The normal range of HRG was established in 126 healthy volunteers. † Values of 23 individuals on stable anticoagulant treatment with a mean of 2.4 International Normalized Ratio, which is slightly higher compared to the intensity of anticoagulation in the proband. ‡ A persistent elevated HRG antigen was confirmed after three

No abnormalities were found in the coagulation assays taking the intensity of oral anticoagulant treatment into account. Protein C, protein S activities and resistance to activated protein C were not determined in the proband who was on stable anticoagulant treatment. To exclude familial type II protein C or protein S deficiency or resistance to activated protein C, these parameters were measured in the spouse, several brothers and sisters and both the children of the proband. No abnormalities were found in the family members investigated. Fibrinolysis parameters were found to be normal in the proband. A minor elevation of u-PA activity was observed.years.

tA persistently elevated level of HRG was found in the proband (161%, and 154% 3 years later). In 9 other family members we also found high levels of HRG up to 209% (see pedigree in figure 1). The mean HRG level of all family members was 124% which was high compared to the mean level found in 126 healthy individuals (99% SD=20%, range: 56%-145%). Possible effects of assortative mating have been excluded in a previous parent-twin study on the heritability of HRG levels (Boomsma et al., 1993). Four women in generation III (III-11, III-15, III-17 and III-18) used oral contraceptives which may lead to a decrease in HRG level of 20%-25% (Jespersen et al., 1990). One of these women (III-17) had an elevated HRG. The other three women had normal HRG and this would not become elevated when a correction of 25% was applied. No corrections were therefore made for the usage of oral contraceptives.

A normal plasminogen and heparin binding of HRG was observed in an affinity crossed immunoelectrophoresis assay with plasma of the proband (data not shown).

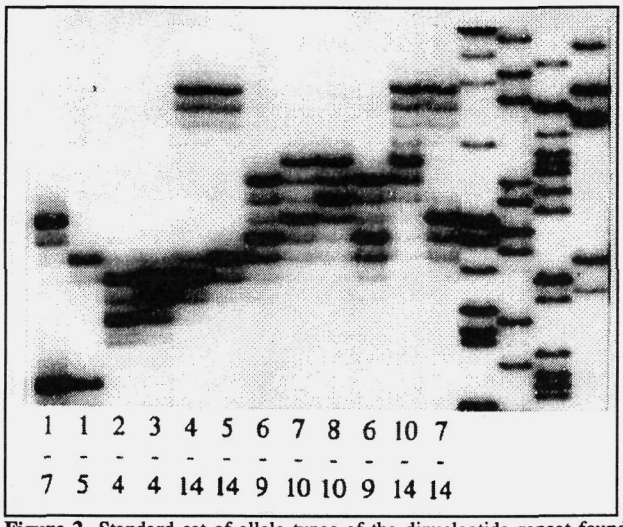

Figure 2. Standard set of allele types of the dinucleotide repeat found in the normal population. Genotypes (allele 1-14) are noted at the bottom.

Dinucleotide repeat genotypes

The genotype with respect to dinucleotide repeat was determined in all family members. Eight different allele types were found and heterozygosity was observed in 30 out of 34 family members (see pedigree in figure 1). A standard set of allele types of the dinucleotide repeat is shown in figure 2. Only in one marriage was information lost due to similar alleles of the dinucleotide repeat in father and mother (II-9 and II-10). No evidence for recombinations or mutations was found within this pedigree.

Linkage

Linkage was performed regarding HRG levels as a quantitative trait with a bimodal distribution caused by two classes. Lod scores and corresponding recombination fractions were calculated assuming a mean level of 99% for the class with normal HRG levels and different mean HRG levels (135%, 145%, 155% and 165%) for the class with high HRG levels.

Maximum lod scores for all analyses were found at Q=0 indicating complete linkage. A maximum lod score of 4.65 at Q=0, representing odds in favour for linkage of 46,000:1, was found at the lowest mean level (135%) for the high-level class. When the mean of the high-level class was set at 145% which was the highest HRG level of the normal range, a lod score of 4.17 was found (odds in favour for linkage 22,000:1). Higher mean values of 155% and 165% both gave lod scores of 2.99 which represent odds in favour of linkage of 1000:1.

In all calculations presented above, a population prevalence of elevated HRG levels of 5% was assumed. However, lod scores did not show substantial changes when the population prevalence was set at 10% or 1% (data not shown).

Dinucleotide repeat genotype and HRG level

Elevated levels of HRG were frequently found in association with family members that carry allele 6 of the dinucleotide repeat polymorphism, indicating that high HRG levels are linked to this allele. HRG levels of individuals with allele 6 were on average higher (149%, n=13) than of individuals without allele 6 (109%, n=25). The mean HRG level of all individuals was 124% (n=34).

The genotype was not in all cases predictive for an elevated HRG level (see pedigree in figure 1). In five family members a normal HRG level was found in combination with allele 6 (III-2, III-8, III-9, III-20, III-21). However, three out of these five individuals did have a HRG level which is above the mean HRG level in the family. An elevated HRG level was found in two individuals that did not carry allele 6: a father (II-12) who was related by marriage and his daughter (III-17).

HRG levels and thrombosis

Of the six family members with a history of thrombosis three members had an elevated HRG level. One individual with a history of thrombosis was related by marriage (II-15) and had no elevated HRG. Both grandparents had thrombosis but

could not be included in the present analysis. Elevated HRG levels were also found in seven family members without a history of thrombosis.

Discussion

In this paper we describe a family with familial thrombosis in which no known risk factors for thromboembolic disease could be found. The only abnormality observed was a high level of HRG. By investigating this family, high levels of plasma HRG were found in 10 out of 34 family members distributed over two generations, suggesting a hereditary nature of this trait.

To investigate the possibility that specific HRG gene variants are associated with elevated plasma HRG levels in our family, we studied the inheritance pattern of a multi-allelic dinucleotide repeat which is located in the intron between the last two exons of the HRG gene (Hennis et al., 1994). Due to the large number of alleles and a high degree of heterozygosity, little information is lost in the pedigree.

Linkage was calculated between the dinucleotide repeat and plasma HRG levels regarding the HRG level as a quantitative trait and assuming a bimodal distribution of HRG levels. Two classes, one with normal levels of HRG (unaffected) and a second with high levels of HRG (affected) were defined by a mean and a variance. Using this approach, lod scores tended to decrease when the mean value for the class with high HRG levels was increased. This can be explained by a reduction of the overlap between the distributions of the two classes. The higher the mean HRG level of the high-level class was chosen, the more family members have a possibility to be assigned to the normal-level class. These individuals are then considered as recombinants which leads to lower lod scores.

By convention a lod score of 3 (odds in favour of linkage of 1000:1) is taken as a threshold for linkage. In the present family we found a maximum lod score of 4.65 and a minimum of 2.99 both at zero recombination, presenting substantial evidence for linkage. This may indicate a significant effect of the HRG locus on plasma HRG levels in general, although it may also be a unique effect in this family. In all calculations a population prevalence of elevated HRG levels of 5% was assumed (Boomsma et al., 1993). However, lod scores did not show substantial changes when the population prevalence was set at 10% or 1%.

The majority of the elevated HRG levels appeared to be coupled to allele 6 of the polymorphism (figure 1). A marked difference was found between the average HRG level of family members with allele 6 and members without allele 6 (149% versus 109%). This result suggests a specific effect of the HRG gene which is coupled to allele 6, on the HRG level. The allele frequency of allele 6 in a group of 130 healthy volunteers is 1.5%. In this group there is no indication that allele 6 is coupled to elevated HRG levels (Hennis, unpublished observations). It seems therefore reasonable to assume that allele 6 itself is not related with raised HRG levels and that

the association between allele 6 and elevated HRG levels is most likely to be purely by linkage within this family.

Not all family members with allele 6 do have elevated HRG levels. It is possible that the lower levels of HRG in these family members are due to neutralizing effects caused by other HRG alleles assuming that a multiallelic HRG locus is present. In one father who is married into the pedigree and his daughter, an elevated HRG level is found in the absence of allele 6 suggesting that the HRG gene associated with elevation of HRG may also be coupled with other alleles of the dinucleotide repeat.
Linkage cannot discriminate between major and minor genes (i.e. the genetic background) that are involved in a given trait. It is therefore not possible to exclude a polygenic model. This may either be a model for a multi-allelic HRG locus or a model involving different loci. In a recent parent-twin study (Boomsma et al., 1993) 69% of the variance in HRG level could be ascribed to genetic background. In such a study design, the dinucleotide polymorphism could be used to determine the contribution of the HRG locus to the heritability.

In theory elevation of plasma HRG levels can be caused by an abnormality in the promoter region or elsewhere in the gene causing overexpression of the protein. An increased level can also be due to a deviating protein structure resulting in either increased synthesis or decreased clearance. In the investigated family no indication was found for the presence of an abnormal HRG molecule. Both the binding of heparin and plasminogen appeared to be normal in an affinity crossed immunoelectrophoresis assay. As yet nothing has been reported about abnormal forms or polymorphisms of the HRG molecule in relation to either high HRG levels or thrombosis.
Another possibility is that the elevation of HRG is age dependent. In this family a statistically significant correlation ($r=0.35$, $p<0.05$) was observed between age and HRG level. A comparable age dependent increase with an even stronger correlation was found in a family described by Hoffmann et al. (1993). Recently, it was also reported that parents have higher HRG levels than their children (Boomsma et al., 1993) but thus far no correlation between age and HRG level has been found in groups of unrelated healthy individuals (Hoffmann et al., 1993). Age may have consequences for linkage since the overlap between the classes of normal and high HRG levels may be greater in the younger generation leading to more false recombinants. However, as the magnitude of the age effect is unknown no corrections for age have been implemented in the linkage analysis of this family.

In a previous report, it is hypothesized that elevated HRG levels may contribute to a prothrombotic state by reducing the availability of plasminogen which can be activated into plasmin (Lijnen et al., 1980). Elevated HRG levels are found in three out of six family members with thrombosis. However, due to the small number of family

members with a history of thromboembolic disease no conclusions can be based on this particular family with respect to a causal relationship between thrombosis and elevation of HRG (see also pedigree in figure 1). To further study this relationship, more families have to be included in future studies. In addition characterization of the HRG gene and of the function of HRG in the haemostatic system are needed to understand the possible contribution of elevated HRG to the prothrombotic state. It is therefore important to screen the HRG gene for functional mutations or polymorphisms in families with high HRG levels.

Acknowledgements

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Chapter 5.2

Aa amino acid polymorphism in histidine-rich glycoprotein (HRG) explains 59% of the variance in plasma HRG levels

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Summary

A pedigree-base maximum likelihood method developed by Lange et al., (1976) was used to study the contribution of a newly defined di-allelic amino acid polymorphism in histidine-rich glycoprotein (HRG) to the plasma levels of HRG. In four families (n=99) selected on the prevalence of elevated HRG and thrombosis, and 20 unselected volunteers we found a heritability of 70%, an age effect of 3% and an effect of individual environmental factors of 27%. These results are remarkably similar to the results found in a previous parent-twin study in which a heritability of 69% and an effect of random environment of 31% was found. The overall genetic influence in the present study can be subdivided into an effect of 59% by the HRG phenotype and 11% by residual genetic factors. The influence of the HRG phenotype of 59% can entirely be explained by adding up the effect of the two alleles that make up the phenotype. These results indicate a codominant inheritance pattern of HRG levels in which the genetic influence can almost completely be ascribed to the additive effect of the di-allelic HRG locus whereas only a small part is due to other loci.

Introduction

The human plasma component histidine-rich glycoprotein (HRG) is a single chain protein which is thought to act as a modulator of coagulation and fibrinolysis. (Koide, 1988b). In a few studies a slightly higher prevalence of elevated HRG plasma levels has been found in groups of patients with venous thrombosis (Samama et al 1983; Engesser et al., 1988; Ehrenforth et al., 1994). In addition, inherited elevation of HRG has been found in several thrombophilic families (Engesser et al., 1987, Angles-Cano et al., 1993, Castaman et al., 1993a). However, it is not clear whether the apparent relationship between elevated HRG levels and thrombosis is causal or coincidental.
Recently, we described a family in which an association was observed between elevated HRG levels and a specific allele of a dinucleotide repeat polymorphism (Hennis et al., 1993). This polymorphism is located between the last two exons of the structural gene for HRG which encloses about 10kb and has lately been localized on chromosome 3q28-q29 (Hennis et al., 1994a). The observed association may indicate that a variant of the HRG gene which is coupled to this specific allele of the dinucleotide repeat polymorphism is responsible for the high HRG levels in this family. However, it is not clear yet whether the gene causing elevated HRG levels is unique to this family or whether it is also present in the population.
The presumed genetic influence on HRG levels was also confirmed in a parent-twin study (Boomsma et al., 1993b). In this study evidence was found for a simple additive genetic model of inheritance in which 69% of the variance in HRG levels could be accounted for by genetic factors. The residual 31% of interindividual variance was due to non-genetic factors unique to each individual. No evidence was found for the influence

of common environment shared by family members. We then suggested that the substantial heritability justifies the search for quantitative trait loci. This is possible if suitable markers are available for the HRG gene or for loci that may influence HRG levels.
Up to now no suitable marker for the HRG locus was available. However, recently we described two common molecular weight variants of HRG. The difference in molecular weight is due to a di-allelic amino acid polymorphism in the second cystatin domain of HRG (Hennis et al., 1994b). The two forms noted as form 1 (77 kDa) and form 2 (75 kDa) appear as a doublet on SDS-PAGE and have frequencies of 0.35 and 0.65, respectively. The three possible phenotypes (homozygous: 1-1 or 2-2 and heterozygous: 1-2) followed a mendelian inheritance pattern. With the availability of this polymorphism (i.e. the phenotype) for HRG it is now possible to discriminate between influences of different alleles at the HRG locus itself and contributions of alleles at other loci.
Recently, we studied four thrombophilic families in which elevated levels of HRG were found in several generations. No relationship was found between HRG levels and thrombosis in these families (Hennis, Boomsma, Engesser and Kluft, submitted). In the present study we used a pedigree-based maximum likelihood method developed by Lange et al. (1976) to study the contribution of the HRG locus to the variance in plasma HRG levels in these four families supplemented with 20 healthy volunteers.

Materials and methods

Subjects

Probands with thromboembolic disease, a persistently elevated HRG level and a family history of thrombosis were selected from a previous study on the relationship between plasma HRG levels and idiopathic thrombophilia (Engesser et al., 1988). Probands were included when at least two other family members had had thromboembolic disease. Initially six probands were found who matched the criteria. Two probands (W.K. and C.B.) were sisters and therefore five independent families were investigated. Finally four families of proband W.K./C.B., F.Z., J.K. and B.Z. enclosing 119 individuals in three generations, were included in the present study. The fifth family was not available for investigation
A persistently elevated level of HRG was found in all five probands (table 1). Pedigrees of the probands are shown in figure 1. In all but one family several individuals were found who have an elevated HRG level (^{3} 145%). In 9 family members of proband B.Z. we found elevated levels of HRG up to 209%, in the family of probands W.K./C.B. four other family members were found and also four family members with elevated HRG were found in the J.K. pedigree.
One of the four families (J.K.) has partly been described by Engesser et al. (1987).

Table 1. HRG levels in plasma of probands at the first sampling and after an asymptomatic period of at least one year.

HRG antigen (%)	Proband W.K.	Proband C.B.	Proband F.Z.	Proband J.K.	Proband B.Z.
First	194	177	165	164	161
Second	181	163	152	150	154

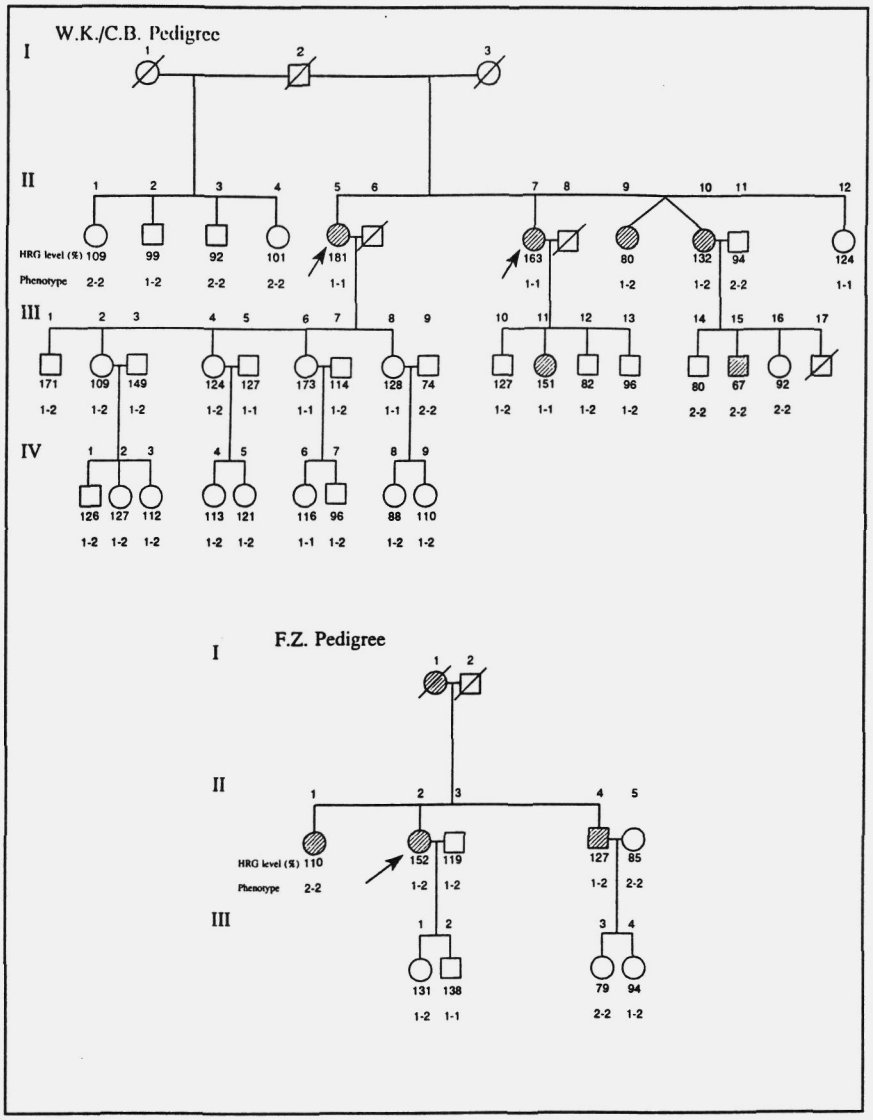
The normal range of HRG was established by taking the extremes found in 126 healthy volunteers: 56%-145%.

Age, gender and HRG levels of volunteers (n=20) were previously described in a study on the longitudinal variation of HRG (de Bart et al., 1992).
This study was approved by the Medical Ethical Committee of HGO-TNO and informed consent was obtained from family members and volunteers.

Blood collection
Blood was obtained from family members and volunteers by a single venipuncture using vacutainer tubes (Becton Dickinson, France) with either sodium citrate or sodium EDTA as anticoagulant. Samples were placed on ice and centrifuged immediately at 3000g for 30 min at 4°C. Plasma was separated and stored at -80°C until use. Samples were thawed only once immediately before measurement.

HRG measurement
Plasma HRG levels were measured by radial immuno diffusion (Mancini et al., 1965) as described previously (Boomsma et al., 1993b). HRG concentrations were calculated from a standard dilution series of pooled plasma obtained from 26 healthy volunteers. The same anticoagulant was not in all families used to prepare plasma. In family W.K./C.B. and in the volunteers sodium citrate was used, in family F.Z., J.K. and B.Z. sodium EDTA was used. To circumvent differences in measurement due to variation in anticoagulant, calibration lines were prepared both from EDTA and citrated pooled plasma. HRG levels were expressed as a percentage of pooled plasma taking citrated pooled plasma as 100%. The inter-assay coefficient of variation of the duplicate measurements was 10%. Using this method HRG levels were determined in 126 healthy

Detection of HRG phenotypes
Polyclonal antibodies from rabbit anti-HRG antiserum raised against human HRG (Behringwerke, Germany) were purified using protein A Sepharose 4B (Pharmacia, Sweden). These antibodies were coupled to CNBr activated Sepharose 4B (5 mg protein per ml gel) according to the manufacturers instructions (Pharmacia, Sweden). For phenotyping, HRG was isolated using a small-scale batch-wise isolation procedure: 300 ml plasma was incubated overnight at 4°C with Sepharose coupled anti-HRG in the presence of 40 KIE aprotinin.


W.K./C.B. Pedigree
I
II
III
IV
HRG level (%)
Phenotype
F.Z. Pedigree

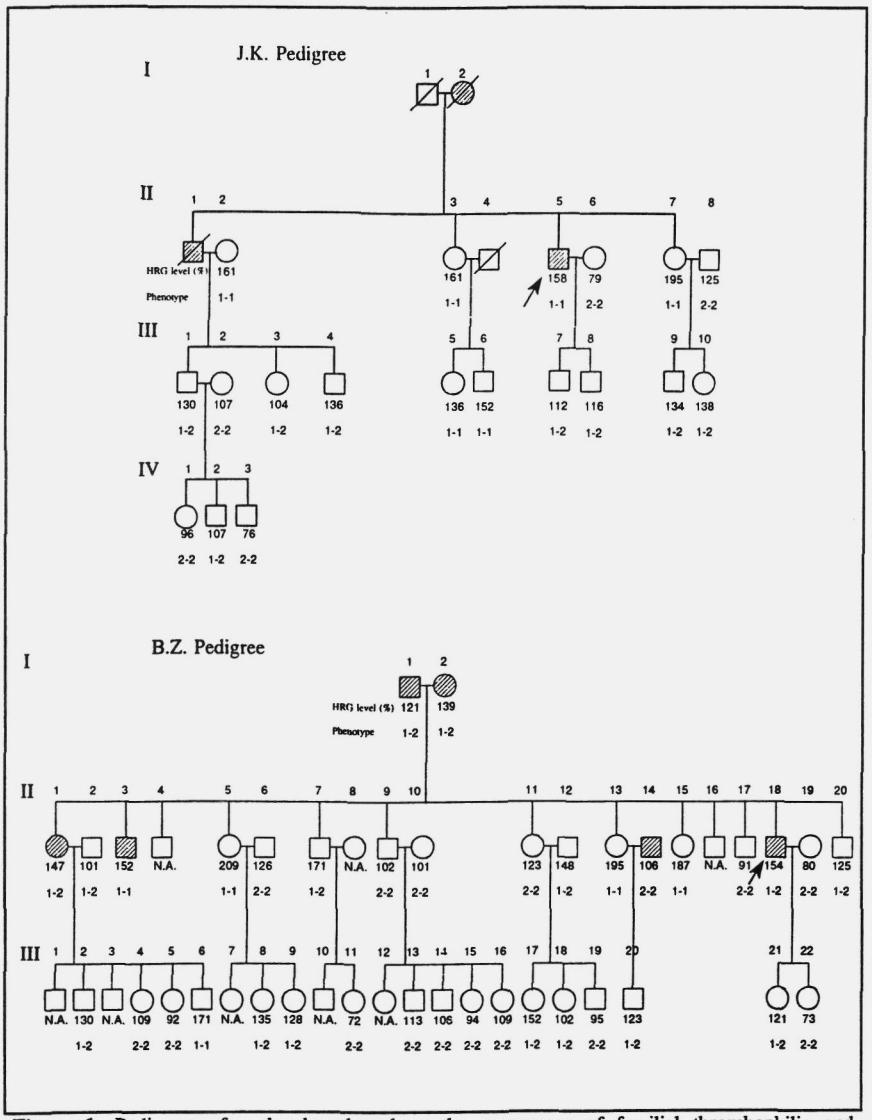


Figure 1. Pedigrees of probands selected on the occurrence of familial thrombophilia and elevation of plasma HRG. Family members not available for investigation are indicated with N.A.. The HRG level and the phenotype are shown below each individual. Probands are indicated by an arrow. Dashed symbols indicate a history of thrombosis. The diagnosis of deep venous thrombosis was required to be confirmed at least once by ascending venography, Doppler ultrasound or impedance plethysmography. The diagnosis of pulmonary embolism had to be confirmed by perfusion lung scanning and the diagnosis of recurrent superficial thrombophlebitis was required to be established by a physician (Engesser et al., 1989). individuals. The mean HRG level was 99% (SD=20%) and the range was 56%-145%. For the selection of the probands HRG levels ^{3} 145% were considered to be elevated.

After washing 3 times with 200 ml 10 mM sodium phosphate, 1 M NaCl, 40 KIE aprotinin, pH 7.4, HRG was eluted with 0.1 M glycine-HCl, pH 2.8. Eluted fractions were neutralized with a half volume of 0.2 M Tris-HCl, 0.005% Tween-20, pH 8.5. Concentrations of HRG in elution fractions were determined by a sandwich ELISA specific for HRG. Polyclonal rabbit anti-HRG antibodies were used for coating. Horseradish peroxidase conjugated rabbit anti-HRG IgG was used as detecting antibody. Colour development was performed using tetramethylbenzidine. Serial dilutions of citrated pooled plasma were used for calibration, assuming a HRG plasma concentration of 100 mg/ml (Lijnen et al., 1980).
Phenotypes of individuals were determined by subjecting 200 ng purified HRG to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) using 10% polyacrylamide gels to separate the two forms. Before electrophoresis, HRG samples were mixed with sample buffer with 2% b-mercaptoethanol as reducing agent and heated at 100°C for 3 min. HRG was detected by immunoblotting. The blotting procedure and subsequent immunostaining were performed as follows: after electrophoresis HRG was blotted onto a PVDF-membrane (Immobilon; Millipore, MA, USA) using a semi-dry blotting system (Pharmacia LKB Biotechnology, Sweden). Blotting was performed for 1.5 hours at 0.8 mA/cm^2 in dry-blot buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol). Following blotting, the membrane was incubated for 1 hour with 3% BSA in washing buffer (150 mM NaCl, 10 mM Tris, 0.05% Tween 20, pH 7.4). The membrane was rinsed with washing buffer and incubated for 1.5 hours with rabbit anti-HRG antibodies (Behringwerke, Germany). Then, the membrane was incubated for 1.5 hours with horse radish peroxidase conjugated goat anti-rabbit antibodies (Nordic, The Netherlands) in washing buffer. Subsequently, the blot was stained by mixing 75 ml staining buffer (200 mM NaCl, 50 mM Tris, pH 7.4) with 15 ml methanol containing 45 mg 4-chloro-1-naphtol and 40 ml 30% peroxide solution. The staining reaction was stopped by rinsing the membrane with water.

Statistics

For statistical analysis we used a pedigree-based maximum likelihood method developed by Lange et al. (1976), in which for a given pedigree of n individuals a vector of observations (x) is defined and a vector of expected values (E(x)), that can depend on measured variables such as sex, age or measured phenotype. The covariances between the residual part of the observations, i.e. the part that is not accounted for by the measured phenotype or other variables, depend on the relationships between the pedigree members and on the genetic model assumed for the observations. Throughout we have modelled the variance in HRG not accounted for by the measured phenotype as consisting of additive genetic and random environmental variance, since we found no evidence for the influence of common environment in a parent-twin study (Boomsma et al., 1993b).

For a given E(x) and expected covariance matrix S, the log-likelihood of obtaining the observation vector x is:

$$L = -0.5\ln|S| - 0.5(x-E(x))'S^{-1}(x-E(x)) + \text{constant};$$

where | | denotes matrix determinant and ' denotes matrix transpose.
The joint log-likelihood of obtaining all pedigrees is the sum of the log-likelihood of the separate pedigrees. Estimation of parameters involves selection of parameter values under a specific model which maximizes the joint likelihood of all pedigrees. The likelihoods obtained for different models can be compared with chi-squared difference tests where $C^2=2(L_1-L_0)$ and L_1 and L_0 denote the log-likelihood for the general (H_1) and the constrained (H_0) hypothesis. The degrees of freedom (df) for this test are equal to the number of independent parameters between H_1 and H_0 (Lange et al., 1976). The FISHER package (Lange et al., 1988) was used for genetic modelling. Ascertainment correction was carried out by conditioning on the probands. The effects of the measured phenotype were first considered by estimating 3 means for the 3 genotypes (2 homozygotes and 1 heterozygote) and alternatively by estimating a mean effect for each of the two alleles and summing the allelic effects. The difference in likelihood (with 1 df) between these two models provides a test of interaction between 2 alleles at the HRG locus (i.e. genetic dominance).

Results

HRG phenotypes

The individual HRG phenotype, the plasma level of HRG and the thrombosis history of family members investigated are shown in the pedigrees in figure 1. The two forms of HRG (1 and 2) that make up the phenotype followed a mendelian pattern of inheritance in all four families. The inheritance pattern strongly suggests that the two protein variants of HRG are encoded by two different alleles of the HRG locus. This is recently confirmed by the elucidation of the underlying molecular defect (Hennis, van Boheemen, Wakabayashi, Koide, Kievit, Dooijewaard, Jansen and Kluft; Manuscript in preparation). The difference in molecular weight is due to a di-allelic amino acid polymorphism in the second cystatin domain of HRG.

Phenotype and HRG level

Descriptive statistics of mean HRG levels and mean ages for each phenotype are presented in table 2. The highest mean levels of HRG were found in individuals homozygous for form 1 and the lowest levels in homozygotes for form 2, whereas heterozygotes had an intermediate HRG level. Frequencies of form 1 and form 2 in this data set were 0.42 and 0.58, respectively. Statistical modelling of the influence of HRG phenotypes on the HRG plasma level was done by considering the phenotypes of individuals of four different families and 20 volunteers. In a strict sense the families were only four independent observations. To take this into account we used a pedigree-

based maximum likelihood method developed by Lange et al. (1988). The statistical analysis of the HRG level is shown in table 3 as log-likelihood estimates for 6 models (as indicated in the legends of table 3). The best model (VI) included contributions of age, alleles 1 and 2, residual genetic factors and random environmental factors.

Table 2. Descriptive statistics of mean HRG levels and mean ages in individuals with the same phenotype (excluding the probands).

Phenotype	Number of individuals	Mean HRG level (%)	Mean Age
1-1	20	156	46
1-2	55	121	37
2-2	39	93	40
All	114	118	40

Table 3. Results of maximum likelihood analysis of HRG levels in four families and 20 volunteers. Log-likelihood estimates for 6 models are shown.

Model	Log-likelihood	Tested against model	Chi-squared difference test	df for difference test
I	-383.02			
II	-383.04	I	0.02	1
III	-388.36	II	10.65*	1
IV	-431.88	II	97.68*	2
V	-389.36	II	12.65*	1
VI	-383.35	II	0.63	1

* significant decrease in likelihood indicating that the effect tested in this model is significant.

Model definition:

I. Most general model allowing for: (i) effect of gender, (ii) effect of age, (iii) differences in mean values of HRG phenotypes, (iv) additive genetic influence and (v) random environmental variability.

II. No gender difference.

III. No age regression.

IV. Means of all phenotypes are the same.

V. No residual genetic variance.

VI. Effect on phenotypic mean is sum of effect of each allele.

Model VI is the best model in this analysis with significant contributions of the factors tested in model III, IV and V. This indicates that there are: (i) no effects of gender, (ii) significant effects of age, (iii) significant differences in mean levels of different phenotypes, (iv) significant effects of residual additive genetic factors (i.e. the contribution of other genes than HRG) and (v) that the effect of the two alleles on the phenotypic mean is additive.

Parameter estimates of the contributing factors from two models (II and VI) are shown in table 4. Percentages of the total variance in HRG level that can be explained by these factors are shown in table 5. Environmental factors explained 27% of the total variance whereas age explained 3%. Genetic factors explained 70% of the variance of which 59% could be ascribed to the phenotype and 11% to other genetic factors. In model VI the effect of the phenotype was considered by estimating a mean effect for each of the two alleles and summing the allelic effects. From this test we conclude that the two alleles at the HRG locus act completely additively.

Table 4. Parameter estimates for model II and VI. Mean levels (in percentage pooled plasma) and standard errors are given for HRG phenotypes and alleles in the different models. Parameter estimates and standard errors are given for the age regression and the variability due to genetic factors other than HRG and environmental factors.

Model II (no sex differences)		Model VI (allelic effects)	
	Mean (s.e.m.)		Mean (s.e.m.)
Phenotype 1-1	140.9 (6.09)	Allele 1	69.4 (2.74)
Phenotype 1-2	106.6 (4.56)	Allele 2	38.1 (2.30)
Phenotype 2-2	77.6 (4.89)		-
	Parameter estimate (s.e.m.)		Parameter estimate (s.e.m.)
Age regression	0.32 (0.09)		0.33 (0.09)
Genetic variability	100.7 (47.0)		95.7 (44.7)
Environmental variability	218.7 (45.0)		224.1 (44.4)

Table 5. Percentage of variance in HRG level explained by contributing factors.

	Variance	Percentage explained
Total variance for HRG	841	100
Variance explained by:		
Age	28	3
HRG phenotype	491	59
Residual genetic factors	96	11
Environmental factors	224	27

Discussion

Recently, we reported on four thrombophilic families in which elevated levels of HRG were found in several generations. However, no relationship was found between HRG levels and thrombosis in these families (Hennis, Boomsma, Engesser and Kluft, submitted). In the present study we used these families and a sample of 20 volunteers, to determine the effect of the HRG locus on HRG plasma levels.

The contribution of genetic and environmental factors to the variance in HRG levels has previously been established in a parent-twin study (Boomsma et al., 1993b). Sixty-nine percent of the variance in HRG levels could be ascribed to genetic factors whereas the residual 31% was explained by individual environmental influences. In addition, significant effects of age and gender were found in this study. No evidence was found for the influence of common environment like a shared household. At that time it was not possible to discriminate between influences of different alleles at the HRG locus itself and contributions of alleles at other loci. This would have been possible if suitable markers had been available for the HRG locus or for other loci that influence HRG levels. However, with the newly defined di-allelic amino acid polymorphism of HRG (Hennis et al., 1994b), we have now been able to study the influence of the HRG locus on plasma HRG levels.

In the present study we found a heritability of 70%, an age effect of 3% and an effect of individual environmental factors of 27%. The results found in the parent-twin study and this study are remarkably similar given the two different approaches. In the parent-twin study an unselected sample of 160 Dutch families consisting of adolescent twins and their parents was used, whereas in this study HRG data from 4 pedigrees selected for familial thrombophilia and elevated HRG levels were employed. It has been suggested that twin studies provide consistently higher estimates of heritabilities than family studies. Our data very convincingly show that this has not always to be the case.

The overall genetic influence of 70% could be split up into an effect of 59% by the two forms of HRG and 11% by residual genetic factors. Two statistical models (II and VI, see table 3) were used to estimate the variance in HRG levels explained by genetic factors. In model II the three possible phenotypes were used whereas in model VI the separate alleles which make up the phenotype were used. The latter model was not significantly different from model II and should therefore be considered as more favoured because one degree of freedom is gained (table 3). Mean levels for each phenotype could be predicted by estimating a mean effect on the HRG level for each of the two alleles and summing the allelic effects (table 4). From this we can deduce that the effects of the two alleles on the measured HRG level is completely additive and that dominance of one of the alleles is excluded. The inheritance patterns of elevated HRG levels observed in several families are therefore unlikely to be dominant as was suggested previously (Engesser et al., 1987; Castaman et al, 1993a; Hoffmann et al.,1993).

The inheritance pattern of HRG levels has to be considered as codominant with a contribution of both alleles which can simply be added up. In addition, both alleles are common alleles and there is a residual variance of 38% (remaining genetic and environmental variance). It is therefore reasonable to expect a normal distribution instead of a bimodal distribution for HRG levels of individuals from the general population. Distributions of HRG indeed resemble a normal distribution although they are slightly skewed to the right. (Lijnen et al., 1981a, Boomsma et al., 1993b; Hoffmann et al., 1993).
The heritability is for the greater part explained by the two forms of HRG. Thus, the di-allelic HRG locus is almost entirely responsible for the genetic influence on the HRG level of an individual whereas only a small part is due to other loci. Whenever deviating HRG levels become important with respect to disease, the phenotype should be determined but attention should also be drawn to individual environmental circumstances which contribute about 30% to the HRG level.

Acknowledgements

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Chapter 6

Plasma HRG levels and thrombosis

Chapter 6

Heritable levels of plasma histidine-rich glycoprotein (HRG) are not associated with thrombosis in four thrombophilic families

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Summary

The apparent relationship between elevated plasma levels of histidine-rich glycoprotein (HRG) and thrombosis was investigated in four thrombophilic families and 20 healthy volunteers. A pedigree-based maximum likelihood method developed by Lange et al. (1976) was used to estimate mean HRG levels in individuals with and without thrombosis and to estimate the contributions of genetic and environmental factors to variation in plasma HRG levels. No significant difference was found between HRG levels in individuals with thrombosis and unaffected individuals, indicating that elevated HRG levels are not related to thrombosis in the individuals investigated. Estimates of genetic and environmental contributions to the HRG level from these family data showed that 5% was explained by age, 49% by additive genetic influences and 46% by random environmental influences. These contributions were not statistically different from those previously found in a parent-twin study, confirming the high heritability of HRG plasma levels.

Introduction

Human histidine-rich glycoprotein (HRG) is a single chain plasma protein which is thought to act as a modulator of coagulation and fibrinolysis. (Koide, 1988). In several studies, a higher prevalence of elevated HRG plasma levels has been found in patients with venous thrombosis compared to a control group of healthy individuals. In these studies 5.9% (n=203) (Engesser et al., 1988), 8.7% (n=104) (Samama et al 1983) and 10.8% (n=695) (Ehrenforth et al., 1993) of the patients had elevated HRG levels.
One of the commonly presented mechanisms for the apparent relationship between elevated HRG levels and thrombosis presumes a decreased fibrinolytic potential due to a reduced availability of plasminogen (Plg) for activation into plasmin (Plm). This hypothesis is based on *in vitro* studies of the interaction of HRG with the high affinity lysine binding site of Plg (Kd = 1mM) which prevents plasminogen from binding to fibrin leading to a hampered activation of Plg (Lijnen et al., 1980). However, as yet there is no experimental evidence showing that this mechanism has any pathophysiological importance.
In 1987 the first thrombophilic family was reported in which an inherited elevation of plasma HRG levels was suspected (Engesser et al., 1987). Since then another five families with thrombophilia and elevated HRG levels have been reported (Falkon et al., 1992; Anglés-Cano et al., 1993; Castaman et al., 1993 (2 families); Hoffmann et al., 1993). In all members of these families, that had a positive history of thromboembolic disease, an elevated level of HRG was found. On the other hand several family members did have an elevation of HRG but no history of thrombosis. A point of criticism about these studies is that the families have been reported as separate cases and that no selection criteria for probands has been reported. In this way it cannot be verified

whether the reported association between elevated HRG and thrombosis in these families is only a matter of chance due to the selection of the probands (Rodeghiero et al., 1993). In a previous study of five probands and 44 family members from five families (Engesser et al., 1988) we found no significant association between elevated HRG levels and thrombosis. In that study a chi-square test was used to test for differences between individuals with and without elevated HRG levels (³ 145%) and thrombosis. However, since data obtained from pedigrees are not independent, this is not an appropriate statistical test for pedigree data. Another disadvantage of the previous approach is that a cut-off point for elevated HRG levels had to be defined. It would be more realistic to consider HRG levels as a quantitative variable.

In the present report the heritability of HRG levels and the association between HRG levels and thrombosis is studied in additional family members from four of the previously studied families (Engesser et al., 1988). Blood samples were newly obtained from all family members. Appropriate statistical methods are used to test for differences between HRG levels of individuals with and without thrombosis in 99 members from these four thrombophilic families supplemented with 20 volunteers. We used the FISHER package (Lange et al., 1988) based on a pedigree-based maximum likelihood method (Lange et al., 1976) for modelling the pedigree data. To avoid bias introduced by the criteria used for selection of the families (e.g. thrombosis and elevation of HRG in the proband) correction was carried out by conditioning on the probands.

Materials and methods

Subjects

Probands with thromboembolic disease, a persistently elevated HRG level (³ 145%) and a family history of thrombosis were selected from a previous study on the relationship between plasma HRG levels and thrombophilia (Engesser et al., 1988). The diagnosis of deep venous thrombosis (DVT) was required to be confirmed at least once by ascending venography, Doppler ultrasound or impedance plethysmography. The diagnosis of pulmonary embolism (PE) had to be confirmed by perfusion lung scanning and the diagnosis of recurrent superficial thrombophlebitis (STP) was required to be established by a physician (Engesser et al., 1989). Probands were included when at least two other family members had had thromboembolic disease. Initially six probands were found who matched the criteria. Two probands (W.K. and C.B.) were sisters and therefore five independent families could be investigated. Finally four families of proband W.K./C.B., F.Z., J.K. and B.Z. were included in the present study. The fifth family was not available for investigation. New plasma samples were obtained from the four families and 99 family members were investigated. One of the four families (J.K.) has partly been described by Engesser et al. (1987).

HRG levels of 10 male and 10 female volunteers were as previously described in a study on the longitudinal variation of HRG (de Bart et al., 1992). Mean age of the volunteers was 35 $\pm$ 9 years (range: 24-58).
This study was approved by the Medical Ethical Committee of HGO-TNO and informed consent was obtained from family members and volunteers.

Blood collection
Blood was obtained from family members and volunteers by a single venipuncture using vacutainer tubes (Becton Dickinson, France) with either sodium citrate or sodium EDTA as anticoagulant. Samples were placed on ice and centrifuged within 30 minutes at 3000g for 30 min at 4°C. Plasma was separated and stored at -80°C until use. Samples were thawed only once immediately before measurement.

Haemostatic assays
Assays of activated partial thromboplastin time, prothrombin time, thrombin clotting time, fibrinogen (Clauss method) and factor VIII coagulant activity were performed according to established procedures. Coagulant activities of factor V and VII were measured by one-stage clotting assays, using factor V and VII deficient plasma and rabbit brain thromboplastin (Simplastin-plus, General Diagnostics, Morris Plains, USA). Protein S antigen was measured by a radioimmunoassay previously described by Bertina et al., (1985). Antithrombin III activity was determined by a spectrophotometric assay (Coatest, Kabivitrum, Sweden). Von Willebrand factor antigen, factor II and factor X antigen, heparin cofactor antigen (Bertina et al., 1987) and protein C antigen (Bertina et al., 1982) were measured by Laurell rocket electroimmunoassay. In patients not using oral anti-coagulants, protein C activity was assessed by a chromogenic assay, according to Bertina et al. (1984).
Plasminogen (Plg) activity was determined on a MLA 1000C (Medical Laboratory Automation, Pleasantville, New York, USA) using a chromogenic assay (Dade®, Baxter, Miami, USA). For calibration Dade® CoagCal®N (Lot. no. 540.046, Baxter, Miami, USA) was used. The assigned value of the plasminogen level in this plasma is 106%. a_2-antiplasmin activity was measured by a chromogenic assay according to Friberger et al. (1978). Tissue-type plasminogen activator (t-PA) activity was measured according to Verheijen et al. (1982). Fibrinolytic activity of the intrinsic plasminogen activating system was determined on fibrin plates, using euglobulin fractions from plasma, prepared in the presence of dextran sulphate, with or without the addition of antibodies against urokinase (Kluft et al., 1984). Plasminogen activator inhibitor (PAI) activity was measured according to Verheijen et al. (1984).

HRG measurement
Plasma HRG levels were measured by radial immuno diffusion (Mancini et al., 1965) as described previously (Boomsma et al., 1993b). HRG concentrations were calculated from a standard dilution series of pooled plasma obtained from 26 healthy volunteers.

The same anticoagulant was not used to prepare plasma in all families. In family W.K./C.B. and in the volunteers sodium citrate was used, in family F.Z., J.K. and B.Z. sodium EDTA was used. To circumvent differences in measurement due to variation in anticoagulant, calibration lines were prepared both from EDTA and citrated pooled plasma. HRG levels were expressed as a percentage of pooled plasma taking citrated pooled plasma as 100%. The inter-assay coefficient of variation of the duplicate measurements was 10%. Using this method HRG levels were determined in 126 healthy individuals. The mean HRG level was 99% (SD=20%) and the range was 56%-145%. For the selection of the probands HRG levels ^{3} 145% were considered to be elevated.

Statistics

For statistical analysis we used a pedigree-based maximum likelihood method developed by Lange et al. (1976), in which for a given pedigree of n individuals a vector of observations (x) is defined and a vector of expected values (E(x)), that can depend on measured variables such as sex, age or thrombosis status. The covariances between relatives for the residual part of the observations, i.e. the part that is not accounted for by age, sex and thrombosis status, depend on the relationships between the pedigree members and on the genetic model assumed for the observations. Throughout we have modelled the residual variance in HRG levels as consisting of additive genetic and random environmental variance, recognizing that the genetic part may also reflect environmental influences shared by family members. However, previously we have found no evidence for influences of shared environment in a parent-twin study on plasma levels of HRG (Boomsma et al., 1993b). For a given E(x) and expected covariance matrix S, the log-likelihood of obtaining the observation vector x is:

$$L = -0.5\ln|S| - 0.5(x-E(x))'S^{-1}(x-E(x)) + \text{constant};$$

where $|\ |$ denotes matrix determinant and ' denotes matrix transpose.

The joint log-likelihood of obtaining all pedigrees is the sum of the log-likelihoods of the separate pedigrees. Estimation of parameters involves selection of parameter values under a specific model which maximizes the joint likelihood of all pedigrees. The likelihoods obtained for different models can be compared with chi-squared difference tests where $C^2=2(L_1-L_0)$ and L_1 and L_0 denote the log-likelihood for the general (H_1) and the constrained (H_0) hypothesis. The degrees of freedom (df) for this test are equal to the number of independent parameters between H_1 and H_0 (Lange et al., 1976). The FISHER package (Lange et al., 1988) was used for modelling the pedigree data. Ascertainment correction was carried out by conditioning on the probands.

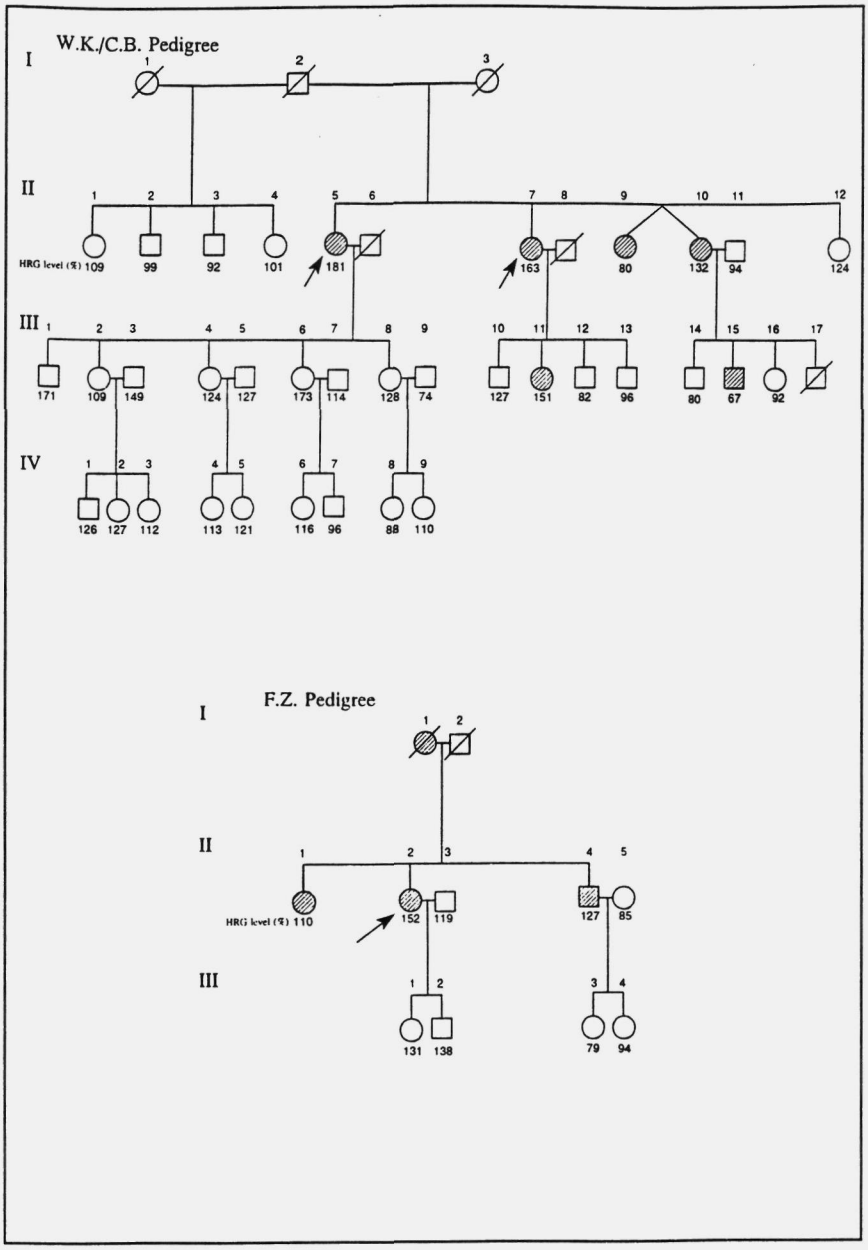
Results

Probands and family members

Proband W.K. (II-5) (see pedigree in figure 1) had superficial thrombophlebitis (STP) after the first parturition at the age of 27. Since then, recurrent STP was diagnosed after

every parturition. At the age of 52 she suffered from an episode of deep venous thrombosis (DVT) and STP in the left leg. Since a sister of proband W.K. was known for a history of thrombosis and since she was also under investigation by the same physician during the selection of the probands, a second proband was defined in this family. This proband C.B. (II-7) had STP after delivery of her second child. A sister of the probands (II-9) had STP in the right leg at the age of 44 and recurrent STP in combination with DVT both in the right leg at the age of 52. Her twin sister had DVT in the left leg after the first parturition at the age of 32 and recurrent STP in both legs after every delivery. The daughter of proband C.B. (III-11) suffered from spontaneous STP at the age of twenty. A son of II-10 (III-15) had a thrombosis in the vena cava inferior at the age of 17.

Proband F.Z. (II-2) suffered from pulmonary embolism (PE) at the age of 27 which was followed by an episode of DVT in the right leg 15 weeks later. At the age of 38 she had recurrent DVT in the left pelvic system. The brother of the proband (II-4) had a postoperative PE whereas the sister (II-1) had postoperative DVT. Furthermore the mother of the proband (I-1) had had thrombosis at a young age but no detailed information on the character of this thrombosis was available.
Proband J.K. (II-5) was described previously by Engesser et al. (1987). The proband had spontaneous DVT in the left leg followed by pulmonary embolism at the age of 52. At the age of 54 the patient suffered from an extensive myocardial infarction. Two other family members had a history of thrombosis. The mother of the proband (I-2) had suffered from recurrent thrombosis after the delivery of her first child at the age of 22. She died at the age of 75 from myocardial infarction. The brother of the proband (II-1) had a spontaneous episode of DVT in the left leg, complicated by pulmonary embolism.
Proband B.Z. (II-18) was a 46-year-old man seen for episodes of recurrent thromboembolism; DVT in the right leg and pulmonary embolism after groin rupture surgery at the age of 32; one year later DVT in the left leg and at the age of 35 a spontaneous thrombosis in the right leg. In all three episodes DVT. The father of the proband (I-1) had DVT in the right leg at the age of 77. The mother (I-2) had DVT in the right leg at the age of 75. One of the proband's sisters (II-1) had recurrent DVT after an operation at the age of 40. A brother of the proband (II-3) had DVT in the right leg at the age of 29 after a trauma. One family member (II-14) who is related by marriage had postoperative DVT in a leg. Another family member (II-12) who is related by marriage was treated with percutane transluminal coronary angiography (PTCA).
In these four families and 20 healthy volunteers a total of 16 individuals with and 103 individuals without a history of thrombosis were investigated.


W.K./C.B. Pedigree
I
II
III
IV
HRG level (%)
109 99 92 101 181 163 80 132 94 124
171 109 149 124 127 173 114 128 74 127 151 82 96 80 67 92
126 127 112 113 121 116 96 88 110
F.Z. Pedigree
I
II
III
HRG level (%) 110 152 119 127 85
131 138 79 94

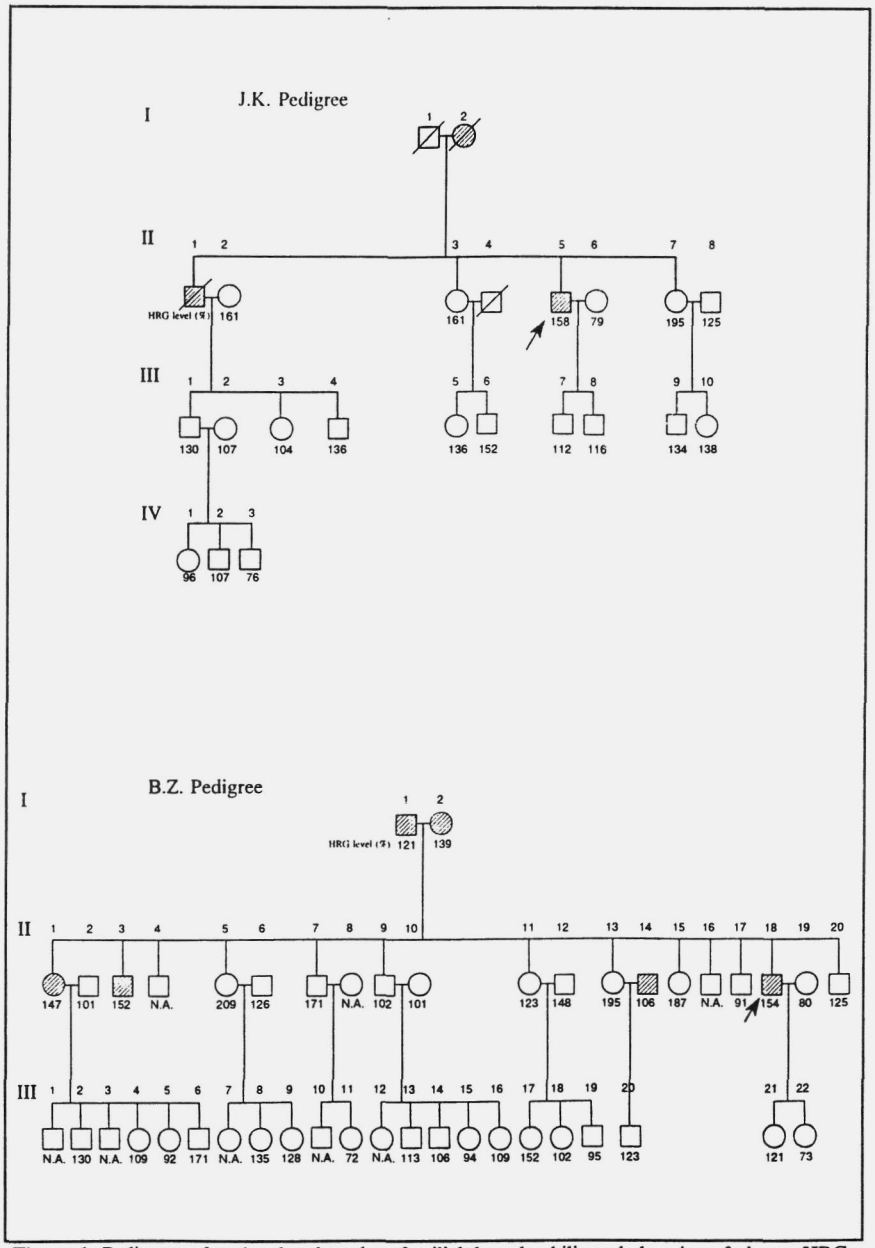


Figure 1. Pedigrees of probands selected on familial thrombophilia and elevation of plasma HRG. Dashed symbols indicate a history of thrombosis. Family members that were not available for investigation are indicated with N.A.. The HRG level is shown below each individual. Probands are indicated by an arrow.

Haemostatic parameters

Probands were extensively studied on coagulation and fibrinolysis parameters in order to exclude the possibility that the apparent familial thrombophilia was due to known or suspected disorders of fibrin formation and fibrinolysis (table 1). From the related probands W.K. and C.B. only proband W.K. was investigated on all haemostatic parameters. No abnormalities were found in the coagulation assays. The activated partial thromboplastin time, prothrombin time, factor VII activity, protein C, protein S and factor II and factor X antigen levels were normal taking the intensity of oral anticoagulant treatment of the probands into account. Thrombin clotting time, fibrinogen, factor V activity, antithrombin III activity and heparin cofactor II were within the normal range. In proband J.K. an elevation of Von Willebrand factor antigen and factor VIII coagulant activity was found. Other family members investigated had normal levels of these factors (Engesser et al., 1988). Von Willebrand factor and factor VIII activity were normal in probands W.K., F.Z. and B.Z. Protein C activity was only determined in proband W.K. and was found to be normal. Protein S activity and resistance to activated protein C were not determined. Therefore, type II protein C and protein S deficiency and resistance to activated protein C were not excluded in these families. Fibrinolysis parameters like plasminogen activity, a_2-antiplasmin activity, t-PA activity, PAI activity, urokinase related fibrinolytic activity and the fibrinolytic activity of the intrinsic

HRG levels

A persistently elevated level of HRG was found in the four probands presented in table 1 and also in proband C.B. who is related to proband W.K.. Pedigrees of the probands are shown in figure 1. In the family of probands W.K./C.B. four other family members were found with elevated HRG (^{3} 145%) whereas in the family of proband F.Z. no other family members have been found with a HRG level ^{3} 145%. Four family members with elevated HRG were found in the J.K. pedigree. In a previously published study on this family one additional member (III-4) had elevated HRG (157%). However, in the plasma sample which was newly collected for the present study, HRG was not elevated anymore (136%) according to the criteria used (^{3} 145%). This indicates that the HRG level of this family member was not persistently elevated. In 9 family members of proband B.Z. we also found elevated levels of HRG up to 209%.
plasminogen activating system were found to be normal in the probands.

HRG level and thrombosis

Statistical modelling of the influence of the HRG plasma level on thrombosis was done by considering the HRG levels of 99 individuals of four different families and 20 volunteers. Results of a maximum likelihood analysis of HRG levels is shown in table 2.

Table 1. Coagulation and fibrinolysis parameters in plasma of probands.

	Proband W.K.	Proband F.Z.*	Proband J.K.*	Proband B.Z.*	Normal range#
Clotting times					
APTT (s)	25	38	28	29	24-33/28-39†
Prothrombin time (s)	11	22	17	17	12-14/17-19†
Thrombin clotting time (s)	16	15	16	16	14.3-18.5
Coagulation factors					
Fibrinogen (mg/ml)	2.2	2.9	3.3	2.2	1.8-4.9
Factor II antigen (%)	110	46	69	57	68-124/46-77†
Factor V activity (%)	108	78	116	100	52-124
Factor VII activity (%)	168	36	54	28	60-157
Factor VIII coagulant activity (%)	188	150	308	172	50-200
Factor X antigen (%)	130	37	50	61	68-124/31-76†
Von Willebrand factor antigen (%)	170	185	240	165	31-216
Antithrombin III activity (%)	97	97	103	91	75-120
Heparin cofactor II antigen (%)	n.d.	90	95	115	61-185
Protein C antigen (%)	140	50	73	55	75-135/40-70†
Protein C activity (%)	128	n.d.	n.d.	n.d.	> 61
Protein S antigen (%)	99	31	84	60	67-125/36-66†
Fibrinolysis factors					
Plasminogen activity (%)	121	108	120	113	70-140
t-PA activity (mIU/ml)	8.4	222	0.6	13.2	> 0
Factor XII-dependent plasminogen activator act. (%)	n.d.	100	133	95	76-158
Urokinase related fibrinolytic act. (%)	n.d.	88	96	88	52-145
Plasminogen activator inhibitor act. (IU/ml)	16.8	4.4	16.4	6.4	1.7-34.0
a_2-antiplasmin activity (%)	127	108	114	95	80-120
HRG antigen (%)‡	194/181	165/152	164/150	161/154	56-145

* Oral anticoagulant treatment at the time of investigation with an intensity of 3.66 (F.Z.), 2.13 (J.K.) and 2.07 (B.Z.) International Normalized Ratio. # Normal ranges were established in at least 42 healthy members of the laboratory staff. The normal range of HRG was established in 126 healthy volunteers. † Values of 23 individuals on stable anticoagulant treatment with a mean of 2.4 International Normalized Ratio. ‡ HRG antigen measurement was repeated after a symptomatic period of at least one year. n.d.= not determined.

From this table we conclude that there is no significant difference between the mean HRG level of individuals with (n=16) and without thrombosis (n=103). The constrained model in which the mean HRG levels of subjects with and without thrombosis were equal, was not significantly different from the general model in which mean HRG levels were allowed to be different in these two groups.

Table 2. Results of maximum likelihood analysis of HRG levels in four families and 20 volunteers. Log-likelihood estimates for 2 models are shown.

Model	Log-likelihood	Tested against model	Chi-squared difference test	df for difference test
I	-431.36			
II	-431.88	I	1.04*	1

* No significant decrease in likelihood indicating that the factor tested in this model has no significant influence on the preceding model. Significance is reached when twice the difference in log-likelihoods of these models is higher than the c^2 corresponding to df=1 and p=0.05 which is 3.84.

Model definition:

I. Most general model allowing for: (i) effect of sex, (ii) effect of age, (iii) differences in mean values of HRG between individuals with and without thrombosis, (iv) additive genetic influence and (v) random environmental variability.

II. Mean HRG level in individuals with and without thrombosis are equal.

Table 3. Parameter estimates and standard errors of factors in the constrained model and percentage of variance in HRG level explained by contributing factors.

	Parameter estimate	Standard error	Variance	% Variance explained
Mean HRG*	93.92	6.735	841	100
Age regression	0.42	0.138	40	5
Residual genetic variance	414.4	170.5	414	49
Residual environmental variance	387.2	121.9	387	46

* The mean HRG is estimated for the whole group. HRG levels for separate groups can be calculated by multiplying the mean age of this group by the age regression and add this to the estimated mean HRG of the whole group. The mean ages for the group with and without thrombosis were 58 and 38, respectively, thus the mean HRG in the group with thrombosis is: (0.42*58) + 93.92 = 118 and in the group without thrombosis it is: (0.42*38) + 93.92 = 110.

Heritability of HRG levels

Parameter estimates of the factors in the constrained model are shown in table 3. In this table we also present the percentage of the total variance in HRG level which can be explained by age, genetic factors and environmental factors. The heritability of HRG levels (i.e. the overall contribution of genetic factors to the variance in HRG levels) appeared to be 49% in this sample of selected families. Residual environmental influences explained 46% of the total variance in HRG levels whereas age explained 5%. No significant influence of sex was found ($c^2(1) < 1$).

Effect of age

In the statistical model presented above it is assumed that the regression coefficients in affected and unaffected individuals are equal. To judge whether this is a reasonable assumption, an overview of mean HRG levels in different age intervals are shown in table 4. The mean HRG levels in this table have been calculated without paying attention to the pedigree structure and should therefore be considered as descriptive data. In separate age intervals of 10 years no differences in mean HRG levels are found between the groups with and without thrombosis (probands excluded), indicating that the age effect is similar in both groups.

Table 4. Mean HRG levels (%) in family members with and without thrombosis grouped in age intervals of 10 years.

Age interval	HRG No thrombosis	HRG Thrombosis	HRG Thrombosis Probands excluded
0-10	121 n=1	-	-
11-20	99 n=11	-	-
21-30	114 n=30	67 n=1	-
31-40	111 n=17	151 n=1	151 n=1
41-50	122 n=22	135 n=4	116 n=2
51-60	139 n=14	140 n=3	131 n=2
61-70	139 n=3	127 n=4	115 n=3
71-80	105 n=5	181 n=1	-
81-90	-	130 n=2	130 n=2
Mean age	37.7 n=103	57.8 n=16	57.2 n=11

Discussion

Up to now it has been uncertain whether the apparent relationship between elevated HRG and thrombophilia in families has any clinical relevance. One of the main reasons for this uncertainty has been that in families with thromboembolic disease and familial elevated HRG levels only a small number of individuals could be studied. Another reason has been that families were mostly reported as separate cases and that no criteria for the selection of probands were reported. In addition, in the family studies reported so far it is not clear whether there were other families in which no association was found. In this way bias may be introduced through the selection of the probands and their families and therefore the observed association of both phenomena may be solely a matter of chance (Rodeghiero et al., 1993).

In the present study we the investigated relationship between HRG levels and thrombosis in 99 family members from four thrombophilic families supplemented with 20 non-thrombotic volunteers. This is the first study in which appropriate statistical methods are used to test for differences between HRG levels of individuals with and without thrombosis in family data. We applied a pedigree-based maximum likelihood method (Lange et al., 1988) to estimate the mean HRG levels in affected and unaffected individuals, and defined strictly uniform selection criteria for inclusion of probands. In addition, this approach gives an estimate of the heritability of HRG levels in the pedigree. In the best fitting model no significant difference was found in mean HRG level of individuals with and without thrombosis indicating that in the four families and twenty volunteers presented here, the HRG level is not likely to be associated with an increased risk of thromboembolic disease.

The best fitting model included age, genetic and random environmental influences as factors which significantly contributed to the variance in HRG level. Apart from a sex effect which was not found in this family sample, these contributing factors were the same as previously reported in parents and twins (Boomsma et al., 1993b). In that study additive genetic influences were estimated to be 69%. In the present study we found a heritability of 49%. However, by fixing the heritability at 69% in the model presented here, there is no significant difference in log-likelihoods (c^2=0.80 for 1 df) indicating that the heritability of 49% found in the family sample is not significantly different from 69%. The percentage of variance explained by environmental factors is also comparable in both studies: 31% in the parent-twin study and 46% here. The heritabilities found in the parent-twin study and this study are remarkably similar given the two different approaches. In the parent-twin study an unselected sample of 160 Dutch families consisting of adolescent twins and their parents was used, whereas in this study HRG data from 4 pedigrees selected for familial thrombophilia and elevated HRG levels were employed.

A small but significant age effect was found both in the parent-twin study and in the present family study. Age explains 5% of the variance and has a regression coefficient of 0.42 indicating that the individual HRG level increases on average 0.42% per year. Age

effects have also been described in a family with an abnormal HRG (HRG Eindhoven; Hoffmann et al., 1993) and in patients with angina pectoris (Thompson et al., 1991). With respect to the present study one could argue that the age effect in patients with thrombosis may be different from that in healthy individuals. However, no differences have been observed in HRG levels when patients and healthy individuals were compared within time intervals of 10 years (table 4) indicating that the age effect is not different in both groups. In addition, reanalysis of data from a large group of patients with venous thrombosis (Engesser et al., 1988) revealed an age regression coefficient of 0.5 ($p=0.0045$) which is very similar to the regression coefficient of 0.48 ($p=0.0085$) found in the present study considering only the healthy individuals. In several studies in which HRG levels of patients with thrombosis were compared to controls, little attention has been paid to differences in age between patients and controls. However, the results of our study indicate that age should be implemented as a covariate in the statistical analysis of such studies or that the groups which are to be compared should be matched for age.

As yet no information is available about the penetrance of thrombosis with respect to elevated HRG levels. Although a reduced penetrance may influence the results of the present study, there are no indications that the lack of association between HRG levels and thrombosis is due to a reduced penetrance. First, several family members with thrombosis have a normal or even low HRG level indicating that elevated HRG is not the only condition for thrombosis (see pedigrees in figure 1). Secondly, in the case of a reduced penetrance, it is expected that high HRG levels (after correction for age) would be observed in those individuals without thrombosis that are still relatively young, because they have not yet developed the disease. These individuals would therefore contribute to a higher mean HRG level in young age groups. This is not observed in the present study.

It is interesting to speculate whether it is possible to extrapolate the results from the present study to other groups of thrombophilic patients with an elevated HRG level, and whether we may conclude from this study that an elevated HRG level is in general not a risk factor for thrombosis. In theory, it is expected that the criteria used for the selection of the probands and families would increase the chance to find a relationship between elevated HRG levels and thrombosis. Thus, when no differences in HRG levels are found between individuals with and without thrombosis in the four families studied here, it is reasonable to assume that also no differences will be found in other groups of patients with thrombosis.
In conclusion, no evidence is found for a relationship between elevated HRG levels and thrombosis. However, to definitively reject the postulated relationship between elevated HRG levels and thrombosis, the results presented here need to be replicated in other studies concerning HRG levels in patient groups or families with thrombosis.

Acknowledgements

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Chapter 7

General discussion

General discussion

Previous studies suggested that elevated levels of plasma HRG are associated with thrombosis. It has been postulated that elevated HRG levels have an antifibrinolytic effect which may contribute to a prethrombotic state. *In vitro* experiments indicate that the antifibrinolytic property of HRG is due to an interference of HRG with plasminogen activation which leads to a reduced plasmin activity. In this thesis we focused on the possible causality of the relationship between elevated HRG levels and thromboembolic disease rather than trying to elucidate the possible underlying mechanism of this relationship.
In theory, there are three explanations for a causal relationship. First, it may be a quantitative (type I) effect in which the elevated concentration of HRG is a risk factor for thrombosis. This may be an elevated concentration of a normal HRG molecule (type I_a) or a deviant HRG molecule with a normal function (type I_b). Secondly, it may be a qualitative effect (type II) in which a functionally abnormal HRG molecule is a risk factor for thrombosis and the increased HRG level is an epiphenomenon. Thirdly, it is possible that the combination of a quantitative and a qualitative effect (high level and abnormal function) causes a higher prevalence of thrombosis (type III).

Up to now the relationship between elevated HRG levels and thrombosis has been studied by measuring antigen levels. With this approach it is not possible to discriminate between the three theoretical possibilities mentioned that may underlie the apparent association between elevated HRG levels and thrombosis. In this thesis we studied the role of elevated HRG levels in thrombophilia by a genetic approach. This genetic approach provided evidence for the occurrence of common variants of the HRG gene and molecule. In addition it provided a firm basis for the study of the apparent association between elevated HRG levels and thrombosis in all theoretical possibilities mentioned above. The following aspects of the HRG gene and protein were studied:

1. Linkage between elevated HRG levels and polymorphic markers for the HRG gene.
2. Abnormalities in the HRG gene or protein.
3. The heritability of HRG plasma levels.
4. Association between HRG levels and thrombosis in families.

1. Linkage

The search for polymorphic markers in or near the HRG locus needed for linkage studies has been quite successful. Two polymorphic markers were found: a KpnI RFLP and a dinucleotide repeat polymorphism (chapter 3.1). The highly polymorphic dinucleotide repeat which is localized in the last intron of the HRG gene, proved to be especially useful for linkage studies (chapter 5.1). First, the dinucleotide repeat was used to incorporate HRG in the genetic linkage map of chromosome three. The genetic and physical localization of HRG to chromosome 3q28-q29 provided additional evidence for

the possible evolutionary relationship between the HRG gene and the genes for a_2-HS-glycoprotein and high molecular weight kininogen, which are also members of the cystatin superfamily of cysteine protease inhibitors (chapter 3.2). Secondly, the dinucleotide repeat was used for linkage in a family in which both elevated HRG levels and thrombosis seemed to be inherited (chapter 5.1). Linkage was found between high HRG levels and a specific allele (6) of the dinucleotide repeat, indicating that the HRG gene coupled to allele 6 is associated with high HRG levels.

2. Molecular variants

The first indication of the possible existence of a variant HRG molecule (HRG Eindhoven) was found in a family with a hereditary increase of plasma HRG (chapter 4.1). The HRG of several family members showed a decreased heparin binding. In most family members this phenomenon seemed to be associated with high HRG levels. The discovery of HRG Eindhoven and the results of the linkage analysis in the family mentioned above, stimulated us to purify HRG of several members of these families. Purification of HRG from family members revealed two forms of HRG which differed about 2 kDa in apparent molecular weight (chapter 4.2). The two forms appeared not to be specific for the families but are commonly encountered in the normal population and have frequencies of 0.35 and 0.65 for form 1 (77 kDa) and form 2 (75 kDA), respectively. By analysis of the genes from individuals homozygous for the molecular variants, five different base pair substitutions were found each of which leads to a different amino acid polymorphism in the HRG protein. The amino acid polymorphism at position 186 in the second cystatin domain (Ser in form 1 and Pro in form 2) is responsible for the difference in apparent molecular weight indicating that the two molecular variants are encoded by different alleles of the HRG gene. The difference may be due to the introduction of an extra attachment site for N-linked carbohydrates at the serine residue of form 1. However, the presence of carbohydrates at this extra attachment site remains to be established. The influence of the other amino acid polymorphisms has not been studied further and is still unclear. It can be speculated that the abnormal heparin binding of HRG Eindhoven may be due to a specific combination of the amino acid polymorphisms but this has not yet been investigated.

Remarkably, the two forms of HRG are associated with differences in plasma HRG levels. Individuals who are homozygous for form 1 have higher HRG levels than individuals homozygous for form 2 whereas heterozygotes have intermediate HRG levels. The molecular variants account for 59% of the heritability of plasma HRG levels and have therefore a strong influence on the plasma level of an individual.

3. Heritability of plasma HRG levels

Factors that contribute to individual plasma HRG levels have been studied quite thoroughly in this thesis. Figure 1 shows a diagram with all factors that contribute to the

HRG level. Plasma levels are for the greater part (70%) determined by genetic factors of which the two molecular variants of HRG take 59% into account (chapter 2.1 and 5.2). The remaining 11% can be ascribed to other genes but it cannot be excluded that additional HRG alleles form part of the remaining random genetic influence. Environmental factors contribute about 27% to the variance in HRG plasma levels. Interestingly, no effect was found of shared environment indicating that all environmental influences are due to individual circumstances. The contribution of environmental factors has been estimated without correction for the use of estrogens. Due to the small number of individuals using estrogens no significant effect on heritability was observed. However, the intake of estrogens appeared to have an important effect on individual HRG levels (chapter 2.2). Estrogens decrease HRG levels in a dose dependent way and therefore influence HRG levels for instance in women using oral contraceptives. Another contributing factor appeared to be age. About 3% of the total variance in HRG levels can be ascribed to age (chapter 5.2). The average age regression is about 0.3% per year. A less important contributing factor is gender. A minor effect of gender has been described in the parent-twin study (chapter 2.1) but was not found in the family study (chapter 5.2).

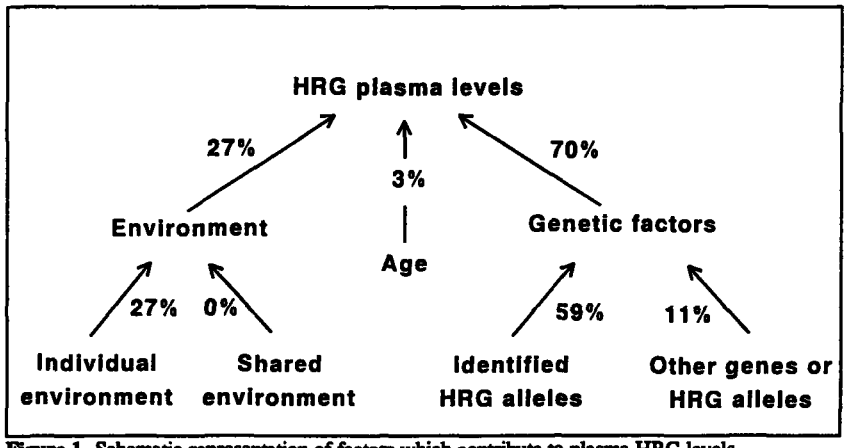

Figure 1. Schematic representation of factors which contribute to plasma HRG levels.

The overall contribution to the plasma HRG level of the alleles encoding form 1 (77 kDa) and 2 (75 kDa) of the HRG protein, is calculated to be 59%. The contribution of both HRG alleles appeared to be additive indicating a codominant pattern of inheritance. Due to the additive character, the HRG level of an individual can partly be predicted by adding up the contributions of the alleles of this individual. The alleles do not contribute in equal amounts to the HRG plasma levels. Allele 1 contributes on average about 70% to the HRG level whereas allele 2 contributes about 40%. Apart from the influences of other genetic factors, age and environment, the HRG level of a homozygote for allele 1 will be 140%, the level of a homozygote for form 2 80% and the level of a heterozygote 110%. As yet it is not known what the exact explanation for this difference is. A

difference in concentration of the two forms may be caused by differences in synthetic rate or in clearance due to the dissimilarity between the two molecules. In theory, it is also possible that the quantification method used shows differences in sensitivity towards the two forms.
Looking back on the results obtained in the linkage analysis, the two forms have also played a role in the association found between allele 6 of the dinucleotide repeat and the high HRG levels in the family described in chapter 5.1. Allele 6 turns out to be coupled to form 1 in this particular family, as can be seen by comparing the results of the linkage analysis with the phenotypes of this family in chapter 5.2. The coupling by itself is not enough to find linkage since form 1 is also coupled to several other alleles of the dinucleotide repeat in this family. However, the circumstances in this family appeared to be good for finding linkage: allele 6 is a rare allele (population frequency is 1%) and the inheritance pattern of allele 6 and form 1 in the family is coincidentally very favourable. Linkage analysis in other families with high HRG levels might not have given a positive lod score due to interference by other haplotypes with respect to the dinucleotide repeat and the two forms of HRG.

4. Histidine-rich glycoprotein and thrombosis

The hypothesis that elevated HRG levels are related to a higher prevalence of thrombosis is based on a few cross-sectional studies in patient groups and several family studies. With the information gathered about the heritability of HRG levels and about the influence of two molecular forms of HRG on the plasma level, it is interesting to discuss the possible role of elevated HRG levels in thrombosis. First, we will discuss the implications, but also the pitfalls of previously reported cross-sectional studies. Then we focus on family studies and discuss the implications and restrictions of the family study described in chapter 6.

Previous studies

Cross-sectional studies. Three cross-sectional studies have been reported in which HRG levels of patients with thrombosis have been compared with a control group of healthy volunteers. In these studies 5.9% (Engesser et al., 1988), 8.7% (Samama et al., 1983) and 10.8% (Ehrenforth et al., 1994) of the patients have HRG levels above the upper limit of the normal range. The most important criticism of the percentage of elevated HRG levels found in these studies is directed towards the definition of the normal range which is derived from control groups. In all three studies it is not reported whether the patient and control groups were properly age and sex matched. Since the age regression coefficient is about 0.3 (chapter 5.2), this may easily lead to a higher number of elevated HRG levels in the patient group compared to the control group, especially when several older patients are present in this group.
In future research it is therefore important to choose a control group that has properly been matched for age and sex with the patient group. Another exclusion criterion in case

control studies of HRG levels is the use of any estrogen preparations. Estrogens decrease HRG levels in a dose dependent way (chapter 2.2).

Family studies. Previously reported family studies in which an association between elevated HRG levels and thrombosis was described have not been conclusive about a possible causal relationship between these two phenomena. This is partly due to the small number of family members in the families studied. Another problem encountered in these association studies is that families were mostly reported as separate cases and it is not clear whether there were other families in which no association was found. That is to say, the selection criteria are not clearly defined. In this way bias may be introduced through the selection of the probands and their families. The observed association of both phenomena may therefore be solely a matter of chance due to the selection method (Rodeghiero et al., 1993). The only information that is obtained from these families so far is that both thrombophilia and HRG levels may have a hereditary character in these families.

Present study

In one study (Engesser et al., 1988) more families were studied but no appropriate statistical methods were used at that time. A better statistical approach is presented in chapter 6 of this thesis where we applied a pedigree-based maximum likelihood method (Lange et al., 1988) to test for differences in HRG levels between family members with and without thrombosis in four selected families. By defining strictly uniform selection criteria for inclusion of probands this statistical approach anticipates bias introduced by (incomplete) proband selection. In addition this approach accounts for the heritability of HRG levels in the pedigree. Using this method we found no difference between the mean HRG level in family members with and without thrombosis. This result indicates that there is no relationship between HRG levels and thrombosis.

It is tempting to speculate about the implications of the results of this family study for the postulated relationship between elevated HRG levels and thrombosis: is it possible to extrapolate these results to other groups of thrombophilic patients with an elevated HRG level? In other words, can we conclude from this study that an elevated HRG level is in general not a risk factor for thrombosis? In theory, it is expected that the criteria used for the selection of the probands and families would increase the chance to find a relationship between elevated HRG levels and thrombosis. Thus, when no differences in HRG levels are found between individuals with and without thrombosis in the four families studied here, it seems reasonable to assume that also no differences will be found in other groups of patients with thrombosis. However, it should be mentioned that the results of the present study may have been influenced by a reduced penetrance of thrombosis associated with elevated HRG levels. Although no indication for a reduced penetrance is found in our family study, it can also not be excluded in this study design. As yet no further information is available about the penetrance.

Future perspectives

Up to now, the role of HRG in thrombosis has only been studied by relating plasma levels of HRG with thrombosis. With respect to this, another question can be asked: what in fact is an elevated HRG level? From the studies on the heritability of HRG levels we learned that 59% of the HRG levels are determined by an additive effect of two HRG alleles which encode two different molecular weight variants of HRG. Each of the alleles has a different contribution to the plasma HRG level. As yet we do not know whether the reason of this difference in contribution is due to differences in concentration or differences in detection of the two HRG variants. In other words, it is hard to judge whether the possible relationship with thrombosis is due to an effect of the HRG level or an effect of the HRG genotype. Therefore, the question about the clinical relevance of elevated HRG levels and thrombosis should better be answered in a different way. For an exact answer it is necessary to circumvent the possibly misleading plasma levels and to couple thrombosis directly to the HRG gene. This can be accomplished by two approaches:

1. In the first approach, the five amino acid polymorphisms, which can simply be determined by PCR and restriction analysis, can be used to determine individual haplotypes. Individual HRG haplotypes can be studied in a large group of patients with thrombosis and in an age and sex matched control group. Accumulation of certain haplotypes in the group of patients may indicate a causal relationship between HRG genes and thrombosis. This approach does not discriminate between a possible monogenetic or polygenetic nature of the relationship between HRG and thrombosis.

2a. In the second approach the possibility of a monogenetic (2a) or bigenetic (2b) relationship between HRG and thrombosis is investigated. This approach uses the haplotypes to perform linkage analysis in a number of thrombophilic families in which no known risk factors for thrombosis are present. Linkage may indicate a causal relationship between certain HRG genes and thrombosis.

2b. Similar (bigenetic) linkage studies can be performed in families with a genetic defect which predisposes them to thrombosis (e.g. protein S or protein C deficiencies or resistance to activated protein C). Linkage studies in these families may indicate that HRG can act as an additional risk factor for thrombosis.

In conclusion, on the basis of the results presented in this thesis the hypothesized relationship between elevated HRG levels and thrombosis has to be rejected. However, additional studies are needed to definitively exclude a relationship. The genetic studies indicate that HRG levels are for the greater part determined by the two alleles of an amino acid polymorphism. As yet it is not possible to judge whether the postulated role of HRG levels in thrombosis is related to the HRG level or to the genotype with respect to the amino acid polymorphism. However, the results from the genetic studies have provided a solid basis and powerful tools to study further the role of HRG in different forms of thromboembolic disease.

Summary

In several reports histidine-rich glycoprotein (HRG) has been postulated as a new risk factor for thrombosis. *In vitro* studies indicate that elevated levels of HRG are possibly associated with lower fibrinolytic activity. In addition, a higher prevalence of elevated plasma HRG levels was found in patient groups with venous thrombosis and familial elevated HRG levels were found in thrombophilic families. However, on the basis of the available observations in patient groups and families it is not possible to discriminate whether the apparent relationship between elevated HRG levels and thrombosis is causal or coincidental. In this thesis the role of HRG in thrombosis is further investigated by a genetic approach. The relationship between HRG and thrombosis is studied by investigating the genetic background of HRG levels in the population and in selected families.

Chapter 1: The introduction gives an overview of all data which have so far been reported on HRG. Structural and functional properties and several biological interactions of HRG are described. Besides the physiological importance of the interaction of HRG with haemostatic parameters, attention is also paid to interesting interactions of HRG in other physiological processes.

Chapter 2: To characterize the contribution of genetic and environmental influences on plasma HRG levels in the general population, the overall heritability of HRG levels has been determined in 160 Dutch twin pairs and their parents (chapter 2.1). Genetic factors explain 69% of the variance in HRG levels. No evidence for the influence of common environment shared by family members was found. Therefore the residual 31% of variance in HRG levels is explained by individual environmental factors. One of the environmental factors which influences plasma HRG levels is the intake of estrogens (chapter 2.2). Pharmacological doses of estrogens reduce plasma HRG levels in a dose-dependent way. The low-dose of estrogens (equivalent of 5 mg ethinyl estradiol (EE)) which is received by postmenopausal women during hormone replacement has no effect on the HRG level. The use of oral contraceptives (30-50 mg EE) reduces the HRG level by 14-24% and the administration of 300 mg EE during hormone therapy in extremely tall prepuberal girls leads to a lowering of the level by 68%. Since in theory the prothrombotic effect of HRG will only be found upon elevation of plasma HRG levels, the role of HRG with respect to the increased risk of thromboembolism during estrogen treatment seems to be minor.

Chapter 3: The HRG locus has been searched for genetic markers to perform mapping and linkage studies. Two markers, a KpnI restriction fragment length polymorphism and a dinucleotide repeat polymorphism were found (chapter 3.1). The highly polymorphic dinucleotide repeat was used to incorporate the HRG locus into the genetic linkage map of chromosome 3q (chapter 3.2). Furthermore HRG was localized to chromosome 3q28-

q29 by *in situ* hybridization. The genetic and physical localization of HRG in close proximity to the genes of kininogen (3q26-qter) and a_2-HS-glycoprotein (3q27-q28) provides further evidence for the possible evolutionary relationship of the HRG gene with the cystatin superfamily of cysteine protease inhibitors.

Chapter 4: The first indication for the possible existence of a variant HRG molecule (HRG Eindhoven) was found in a family with a hereditary increase of HRG (chapter 4.1). The HRG of several family members showed an abnormal heparin binding. In most family members this phenomenon seemed to be associated with high HRG levels.
The discovery of HRG Eindhoven and the results of the linkage analysis in the family described in chapter 5.1, stimulated us to purify HRG of several members of these families. Purification of HRG from these individuals revealed two forms of HRG which differed about 2 kDa in apparent molecular weight (chapter 4.2). The two forms appear not to be specific to these families but are commonly encountered in the normal population and have frequencies of 0.35 and 0.65 for form 1 (77 kDa) and form 2 (75 kDa), respectively. Genetic analysis of the genes encoding for the two forms reveal that an amino acid polymorphism at position 186 in the second cystatin domain (Ser in form 1 and Pro in form 2) is responsible for the difference in apparent molecular weight. The difference may be caused by the introduction of an extra attachment site for N-linked carbohydrates at the asparagine residue (184) of form 1. However, the presence of carbohydrates at this extra attachment site remains to be established. Four additional amino acid polymorphisms were observed: Ile/Thr (162), His/Arg (322), Arg/Cys (430) and Asn/Ile (475). The possible role of these amino acid polymorphisms is still unclear. The abnormal heparin binding of HRG Eindhoven may also be related to one of the five amino acid polymorphisms but this has not yet been investigated.

Chapter 5: The relationship between different alleles of the HRG locus and plasma HRG levels was studied using the dinucleotide repeat polymorphism (chapter 3.1) and the di-allelic amino acid polymorphism (chapter 4.2). First, the dinucleotide repeat polymorphism was used to study linkage in a thrombophilic family with familial elevation of plasma HRG (chapter 5.1). Linkage was found between high HRG levels and a specific allele (no.6) of the dinucleotide repeat, indicating that the HRG gene coupled to allele 6 of the dinucleotide repeat is associated with high HRG levels in this family.
Secondly, a pedigree-based maximum likelihood method was used to study the contribution of the two forms of the di-allelic amino acid polymorphism to the plasma levels of HRG (chapter 5.2). In four families (n=99) selected on the presence of elevated HRG and venous thrombosis, and 20 unselected volunteers we found a heritability of 70%, an age effect of 3% and an effect of individual environmental factors of 27%. These results are remarkably similar to the results found in the parent-twin study (chapter 2.1) in which a heritability of 69% and an effect of random environment of 31% was found. The overall genetic influence in the present study can be subdivided

into an effect of 59% by the HRG phenotype and 11% by residual genetic factors. The influence of the HRG phenotype of 59% can entirely be explained by adding up the effect of the two alleles that make up the phenotype. The alleles do not contribute in equal amounts to the HRG plasma levels. Allele 1 contributes on average about 70% to the HRG level whereas allele 2 contributes about 40%. Apart from influences of other genetic factors, age and environment, the HRG level of a homozygote for allele 1 will be 140%, the level of a homozygote for form 2 80% and the level of a heterozygote 110%. As yet it is not known what the explanation for this difference is. A difference in concentration of the two forms may be caused by differences in synthetic rate or in clearance due to the dissimilarity between the two molecules. In theory, it is also possible that the quantification method used shows differences in sensitivity towards the two forms. The two forms have also played a role in the association found between allele 6 of the dinucleotide repeat and the high HRG levels in the family described in chapter 5.1. Allele 6 turns out to be coupled to form 1 in this particular family. This may for the greater part explain the linkage found in this family.

Chapter 6: The apparent relationship between elevated plasma levels of HRG and venous thrombosis was investigated in the same data set of four thrombophilic families and 20 healthy volunteers which was investigated in chapter 5.2. The pedigree-based maximum likelihood method was now used to estimate mean HRG levels in individuals with (n=16) and without thrombosis (n=103). No significant difference was found between HRG levels in individuals with thrombosis and unaffected individuals, indicating that elevated HRG levels are not related to thrombosis.
In theory, it is expected that the criteria used for the selection of the probands and families would increase the chance to find a relationship between elevated HRG levels and thrombosis. Thus, when no differences in HRG levels are found between individuals with and without thrombosis in the four families studied here, it seems reasonable to assume that also no differences will be found in other groups of patients with thrombosis. However, it should be mentioned that the results of the present study may have been influenced by a reduced penetrance of thrombosis associated with elevated HRG levels. Although no indication for a reduced penetrance is found in our family study, it can also not be excluded in this study design. As yet no further information is available about the penetrance.
In conclusion, the results presented in this thesis indicate that the postulated relationship between elevated HRG levels and venous thrombosis has to be reconsidered. Firstly, in individuals with and without thrombosis no difference in HRG level was found. Secondly, HRG levels are for the greater part determined by the two alleles of an amino acid polymorphism. It is therefore as yet not possible to judge whether a possible role of HRG levels in thrombosis is related to the HRG level or to the genotype with respect to the amino acid polymorphism. However, the results from the genetic studies presented here provide a solid basis and powerful tools to study further the role of HRG in different forms of thromboembolic disease.

Samenvatting

Op grond van de resultaten uit verschillende klinische studies is gesuggereerd dat een verhoogd plasma gehalte van histidine-rijk glycoproteïne (HRG) een risicofactor voor trombose kan zijn. Deze hypothese wordt ondersteund door *in vitro* experimenten waarin verhoogde concentraties van HRG een remmende werking op de fibrinolyse hebben. Bovendien worden hoge plasma gehaltes van HRG vaker waargenomen in patiënten met trombose dan in gezonde vrijwilligers. Daarnaast zijn er gevallen bekend van families waarin familiale trombose voorkomt in combinatie met verhoogde HRG niveaus. Het is op basis van de waarnemingen tot nu toe echter niet mogelijk geweest om een eventueel causaal verband tussen verhoogde HRG gehaltes en trombose te onderscheiden van een toevallig verband. In dit proefschrift wordt de rol van HRG gehaltes bij trombose nader onderzocht. In de populatie en in geselecteerde families worden genetische- en omgevingsfactoren bestudeerd die van invloed zijn op het HRG niveau in plasma. Vervolgens wordt gekeken naar de relatie tussen HRG gehaltes en trombose.

Hoofdstuk 1: In de introductie wordt een overzicht gegeven van de bestaande literatuur op het gebied van het HRG onderzoek. Zowel structurele als functionele aspecten van HRG komen aan de orde. Naast de fysiologisch mogelijk belangrijke interacties van HRG met hemostase factoren worden ook interessante interacties van HRG in andere fysiologisch processen behandeld.

Hoofdstuk 2: De invloed van erfelijke eigenschappen en omgevingsfactoren op het HRG plasma gehalte is bestudeerd in 160 Nederlandse tweelingen en hun ouders (hoofdstuk 2.1). Genetische factoren blijken 69% van de variantie in HRG gehaltes te bepalen. Omdat de invloed van gemeenschappelijke omgevingsfactoren, zoals het opgroeien in hetzelfde gezin, te verwaarlozen is kan de resterende variantie worden toegeschreven aan individuele omgevingsfactoren. Het gebruik van oestrogenen is een van deze omgevingsfactoren die plasma HRG gehaltes kan beïnvloeden (hoofdstuk 2.2). Onder invloed van farmacologische doses oestrogenen word een doses afhankelijke verlaging van het HRG gehalte waargenomen. Een lage doses (het equivalent van 5 mg ethinyl estradiol (EE)), zoals die wordt gegeven aan postmenopauzale vrouwen om voor een tekort aan oestrogenen te compenseren, heeft geen effect op het HRG gehalte. Het gebruik van orale anticonceptiva (30-50 mg EE) daarentegen doet het HRG niveau met 14-24% dalen. Een nog grotere doses van 300 mg EE, die wordt voorgeschreven om overmatige lengtegroei bij prepuberale meisjes af te remmen, geeft een verlaging van het HRG niveau van 68%. Een ongewenste bijwerking van het gebruik van oestrogeen is de toename van de incidentie van trombose. Een additionele rol van HRG hierin lijkt echter niet voor de hand te liggen, immers het protrombotische effect van HRG wordt in theorie alleen verwacht bij een verhoging van het plasma gehalte.

Hoofdstuk 3: Om het HRG gen te kunnen lokaliseren en om ook koppelingsstudies te kunnen doen is er in het HRG gen gezocht naar polymorfe genetische merkers (hoofdstuk 3.1). Er zijn twee merkers gevonden, te weten een KpnI restrictie fragment lengte polymorfisme en een polymorfe di-nucleotide repeat (een repeterende sequentie van twee baseparen, bijvoorbeeld CA). Met behulp van de hoog polymorfe di-nucleotide repeat kon het HRG gen in de genetische koppelingskaart van chromosoom 3q worden geplaatst (hoofdstuk 3.2). Daarnaast is het HRG gen ook fysisch gelokaliseerd op chromosoom 3q28-q29 met behulp van *in situ* hybridisatie. Het HRG gen blijkt dichtbij twee andere genen te liggen die behoren tot de cystatine superfamilie van cysteïne protease remmers, namelijk kininogeen (3q26-qter) en a_2-HS-glycoprotein (3q27-q28). Met deze genetische en fysische lokalisatie van HRG wordt het mogelijk evolutionaire verwantschap van HRG tot de cystatine superfamilie bevestigd.

Hoofdstuk 4: Een eerste aanwijzing voor het bestaan van een mogelijk abnormaal HRG molecule (HRG Eindhoven) is gevonden in een familie met een erfelijk bepaald verhoogd plasma HRG niveau (hoofdstuk 4.1). Dit HRG molecule vertoond een lagere affiniteit voor heparine en lijkt in de meeste familieleden te zijn gekoppeld aan een verhoogd HRG plasma gehalte. De ontdekking van HRG Eindhoven en ook de resultaten van de koppelingsanalyse die wordt beschreven in hoofdstuk 5.1 gaf aanleiding om het HRG van enkele leden van deze families te zuiveren. In verschillende leden van beide families bleken twee vormen van HRG voor te komen die ongeveer 2 kDa in moleculegewicht van elkaar verschillen. De twee vormen blijken niet uniek te zijn voor deze twee families maar worden ook gevonden in de populatie, en wel met frequenties van 0.35 (vorm 1, 77 kDa) en 0.65 (vorm 2, 75 kDa). Met behulp van moleculair biologische technieken zijn de genen die voor de twee vormen coderen geanalyseerd. Het verschil in moleculegewicht blijkt te worden bepaald door een aminozuur polymorfisme op positie 186 in het tweede cystatine domein. Dit polymorfisme geeft een serine in vorm 1 en een proline in vorm 2. Met de serine in vorm 1 wordt op de arginine op positie 184 een nieuwe bindingsplaats voor N-gebonden suikergroepen gevormd. Deze extra suikergroep zou heel goed het grotere moleculegewicht van vorm 1 kunnen veroorzaken. Het is op dit moment echter nog niet duidelijk of er daadwerkelijk sprake is van een extra gebonden suikergroep. Gedurende de analyse van de genen werden nog vier andere aminozuur polymorfismen gevonden: Ile/Thr (162), His/Arg (322), Arg/Cys (430) and Asn/Ile (475). De rol van deze aminozuur polymorfismen is nog onduidelijk. Het is mogelijk dat de abnormale heparine binding van HRG Eindhoven het gevolg is van één of een combinatie van deze polymorfismen, maar dit is tot nu toe nog niet onderzocht.

Hoofdstuk 5: De relatie tussen verschillende allelen van het HRG gen en plasma HRG gehaltes is onderzocht met behulp van het dinucleotide repeat polymorfisme dat is beschreven in hoofdstuk 3.1 en het aminozuur polymorfisme uit hoofdstuk 4.2. Het dinucleotide repeat polymorfisme is gebruikt voor een koppelingsanalyse in een familie met zowel familiale trombofilie als familiaal verhoogde HRG niveaus (hoofdstuk 5.1). In

deze familie werd koppeling gevonden tussen de verhoogde HRG gehaltes en een specifiek allel van het polymorfisme (allel no.6). Dit kan duiden op het voorkomen van een HRG gen dat een verhoogd HRG gehalte veroorzaakt en dat in deze familie is gekoppeld aan allel 6 van het polymorfisme.
Het aminozuur polymorfisme is gebruikt om de bijdrage van de twee HRG vormen (lees ook: allelen) aan de HRG plasma niveaus te bepalen (hoofdstuk 5.2). Hierbij is gebruik gemaakt van een statistische methode die ontwikkeld is voor de analyse van gegevens verkregen uit families (pedigree-based maximum likelihood method). Vier families (n=99) waarvan de probandi waren geselecteerd op verhoogd HRG en veneuze trombose, en 20 gezonde vrijwilligers werden in de analyse betrokken. Het totaal van erfelijke factoren blijkt in deze studie 70% van de variantie in HRG gehaltes te kunnen verklaren. Leeftijd verklaard 3% en individuele omgevingsfactoren verklaren de resterende 27% van de variantie in HRG niveaus. Deze resultaten zijn bijna gelijk aan de eerder gevonden resultaten in de tweelingstudie (hoofdstuk 2.1). In die studie kon 69% van de variantie In HRG gehaltes worden toegeschreven aan erfelijke factoren en 31% aan omgevingsfactoren. In de huidige studie kunnen de genetische factoren worden onderverdeeld in een bijdrage van 59% door de twee vormen van HRG en een bijdrage van 11% door andere genetische factoren. De bijdrage van het HRG fenotype aan het HRG gehalte kan volledig verklaard worden met het optellen van de effecten van de beide afzonderlijke allelen. Hierbij moet worden opgemerkt dat beide allelen in verschillende mate bijdragen aan het plasma HRG niveau. Allel 1 draagt gemiddeld 70% bij aan het plasma HRG gehalte terwijl allel 2 gemiddeld 40% bijdraagt. Als de invloeden van leeftijd, omgevingsfactoren en resterende genetische factoren buiten beschouwing worden gelaten kan het HRG gehalte in plasma van een individu worden berekend. Het HRG gehalte van een individu homozygoot voor allel 1 is dan 140%, dat van een heterozygoot 110% en dat van een homozygoot voor allel 2 80%. Het is op dit moment nog niet duidelijk waarom de twee allelen een zo verschillende bijdrage leveren aan het HRG niveau. Het is mogelijk dat er een verschil in synthese of klaring van het HRG een rol speelt, maar het kan ook zijn dat de detectie methode een verschillende gevoeligheid voor beide vormen heeft.
De twee vormen van HRG hebben achteraf gezien ook een belangrijke rol gespeeld bij de associatie die gevonden is tussen allel 6 van de dinucleotide repeat en de hoge HRG gehaltes in de familie beschreven in hoofdstuk 5.1. De gevonden koppeling kan grotendeels worden toegeschreven aan het feit dat allel 6 in deze familie is gekoppeld aan vorm 1, de vorm die een hoger HRG gehalte tot gevolg heeft.

Hoofdstuk 6: Dezelfde gegevens van de vier families en 20 vrijwilligers (hoofdstuk 5.2) zijn ook gebruikt om de vermoede relatie tussen HRG gehaltes en veneuze trombose te onderzoeken. De pedigree-based maximum likelihood method werd in dit geval gebruikt om het gemiddelde HRG gehalte te schatten in individuen met (n=16) en zonder trombose (n=109). Met behulp van deze methode werd er geen verschil in gemiddeld HRG gehalte gevonden tussen individuen met trombose en gezonde individuen. Hieruit

kan geconcludeerd worden dat er geen relatie is tussen verhoogde HRG niveaus en trombose. Theoretisch gezien kan er verwacht worden dat de criteria die zijn gebruikt bij de selectie van de probandi juist de kans op het vinden van een relatie tussen verhoogde HRG gehaltes en trombose vergroten. Wanneer er nu geen verschillen in HRG gehalte worden gevonden tussen individuen met en zonder trombose in de families zoals hier boven beschreven, lijkt het redelijk te veronderstellen dat een dergelijk verband ook niet zal bestaan in andere groepen van trombose patiënten. Hierbij dient evenwel opgemerkt te worden dat een mogelijke relatie tussen verhoogd HRG en trombose niet in alle gevallen tot uiting hoeft te komen. Als gevolg van slechts een gedeeltelijke penetrantie van trombose veroorzaakt door verhoogd HRG kunnen de resultaten van onze studie beïnvloed zijn. Alhoewel er in onze studie geen duidelijke redenen zijn om aan te nemen dat er sprake is van gedeeltelijk penetrantie, kan dit niet worden uitgesloten in een dergelijke studieopzet. Additionele informatie uit andere studies over de penetrantie is echter niet voorhanden.

Resumerend kan worden vastgesteld dat de resultaten in dit proefschrift aanleiding geven tot twijfel aan de hypothese waarin een verband tussen verhoogde plasma HRG gehaltes en veneuze trombose wordt voorgesteld. Ten eerste worden geen verschillen in HRG niveaus gevonden tussen individuen met en zonder trombose. Ten tweede worden HRG gehaltes grotendeels bepaald door de twee allelen van een aminozuur polymorfisme. Hierdoor is het niet goed mogelijk om te bepalen of de voorgestelde rol van HRG bij het ontstaan van trombose moet worden toegeschreven aan het HRG gehalte in het plasma van een individu of aan het fenotype van het aminozuur polymorfisme van het individu. De resultaten van de genetisch studies die in dit proefschrift zijn beschreven zijn evenwel een goede basis om een mogelijke rol van HRG in ook in andere vormen van trombose te onderzoeken.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 10 oktober 1964 te Utrecht.
Hij volgde zijn middelbare schoolopleiding aan het Ichthus College te Drachten en behaalde in 1983 het diploma Gymnasium b.
In datzelfde jaar werd begonnen met de studie scheikunde aan de Rijksuniversiteit te Groningen. Na het afronden van de propaedeuse werd biochemie als afstudeerrichting gekozen. De doctoraalfase omvatte een hoofdvakstage bij de vakgroep Biochemie te Groningen onder leiding van Dr. G. AB. Tijdens de stage werd de oestradiol gereguleerde expressie van het kippe vitellogeninegen bestudeerd. Het bijvak werd ingevuld met een onderzoek naar de maatschappelijke aspecten van de toepassing van moderne biotechnologie bij de produktie van voedingsmiddelen. Dit onderzoek werd uitgevoerd bij de het Rathenau Instituut (toen NOTA geheten, Nederlandse Organisatie voor Technologisch Aspectenonderzoek) onder leiding van Dr. ir. L. Sterrenberg (Rathenau Instituut) en Prof. dr. Ph.J. Vergragt (vakgroep Wetenschap en Samenleving, Groningen). Het doctoraalexamen werd in 1989 behaald.
In november 1989 begon de auteur aan het promotieonderzoek dat in dit proefschrift wordt beschreven. Als assistent in opleiding was hij in dienst van de faculteit Geneeskunde aan de Rijksuniversiteit te Leiden en gedetacheerd bij het Gaubius Laboratorium TNO-PG te Leiden. Het onderzoek werd gesubsidieerd door de Trombosestichting Nederland en stond onder leiding van Prof. dr. C. Kluft.
Vanaf oktober 1994 is hij als postdoctoraal medewerker werkzaam op de afdeling Vaat- en Bindweefselonderzoek van het Gaubius Laboratorium TNO-PG. In het kader van een subsidie van het Koningin Wilhelmina Fonds doet hij onder leiding van Dr. J.H. Verheijen onderzoek naar de rol van plasminogeen-activatoren bij het metastaseren van tumorcellen.

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