Hyperhomocysteinemia and venous thrombosis : studies into risk and therapy

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Contents

Chapter 1

Introduction

Introduction

Thrombosis is the term used for pathological formation of blood clots in the venous or arterial vasculature. Self-limited clot formation prevents excessive blood loss and illustrates the natural response to vascular injury. Pathologic clot formation (thrombosis) can represent itself in arterial vessels as arterial thrombosis or in venous vasculature as venous thrombosis.

The hemostatic system includes several major components that regulate the process of clot formation: vascular endothelium, procoagulant plasma protein factors, platelets, anticoagulant proteins, fibrinolytic proteins and antifibrinolytic proteins. All these factors need to be present adequately to regulate clot formation in a way that clots can be formed when needed but excessive clot formation will be prevented.

Venous thrombosis is a common disease. It affects 1-2 individuals per 1000 each year. The incidence is age-related with an increasing incidence by age. Venous thrombosis mostly presents itself as deep vein thrombosis in the legs or pulmonary embolism. Less frequent localizations of venous thrombosis are cerebral veins, arm veins, mesenterical veins and Budd-Chiari syndrome due to thrombosis in the liver veins. The pathophysiology of venous thrombosis is considered to be multicausal[1]. Well- known clinical factors associated with venous thrombosis are pregnancy, malignant disease, prolonged bed rest and major surgery. Over the years several factors have been discovered that contribute to venous thrombosis, both inherited (i.e., Antithrombin deficiency, Protein S or C deficiency) and acquired factors. In the past few years several new risk factors were identified. These include Factor V Leiden, high levels of clotting factor VIII, IX or XI, fibrinogen and the presence of lupus anticoagulant (in combination with anti Beta2-glycoprotein I antibodies)[2,3]. A reduced activity of the fibrinolytic potential may also be associated with venous thrombosis[4]. A combination of clinical factors and clotting abnormalities may further increase the risk for venous thrombosis such as cancer in combination with factor V Leiden or the prothrombin 20210A mutation[5]. Interestingly, factors that are associated with a first event of venous thrombosis appear not invariably associated with recurrent venous thrombosis[6].

Another recently identified risk factor is mild hyperhomocysteinemia. The amino-acid homocysteine was described in the early 1960s and found in high concentrations in the urine of children with ‘homocystinuria’ (i.e., high concentrations of homocystine, a disulfide of homocysteine, in the urine)[7]. Classic homocystinuria is an ‘inborn error of metabolism’, caused by a homozygous deficiency of the enzyme cystathionine-β-synthase, an enzyme involved in the transsulphuration of homocysteine to cysteine. Patients with this disease suffer from ectopia lentis, a Marfanoid posture, mental retardation and osteoporosis. Additionally, in the second and third decade of life, they have a

risk of 40 to 50% percent of developing arterial and venous thrombosis. This high incidence of vascular disease led to the hypothesis that high homocysteine concentrations due to decreased activity of cystathionine-β-synthase might be involved in the pathogenesis of vascular disease in individuals not affected with homocystinuria. It was first suggested that the activity of cystathionine-β-synthase was decreased in patients with arterial vascular disease[8,9]. In later studies this finding could not be reproduced[10,11]. Moreover, carriers of the mutation in the cystathionine-β-synthase gene did not have an increased risk for developing vascular disease. Nevertheless, homocysteine levels were elevated in patients with vascular disease in comparison with those without vascular disease, as was observed in case-control studies and prospective investigations. These studies mostly confirmed the hypothesis that homocysteine is associated with arterial vascular disease[12,13].

We were among the first to describe an association between venous thrombosis and hyperhomocysteinemia in patients with recurrent venous thrombosis[14], and subsequently demonstrated an increased risk for a first deepvenous thrombosis in the so-called Leiden Thrombophilia Study[15]. A recent meta-analysis confirmed the association between hyperhomocysteinemia and venous thrombosis in both case-control studies and prospective studies[16].

This thesis focuses on the relation between plasma homocysteine levels and venous thrombosis and deals with several aspects of this relation. The main investigation in this thesis is based on two starting points.

First, hyperhomocysteinemia is associated with venous thrombosis but a causal relationship has not been proven. Observational studies may have showed associations that were either the result of thombosis having an effect on homocysteine levels when blood samples are taken after the event, or arise from a true cause of thrombosis also affecting homocysteine levels. The second starting point is that homocysteine can be easily lowered. A combination of folic acid, a synthetic form of folate, hydroxycobalamin and pyridoxine decreases homocysteine concentrations by approximately 35% in both thrombosis patients and healthy persons with hyperhomocysteinemia, but also by 20-30% in patients and healthy persons with normal homocysteine plasma concentrations[17].

These two points lead to the possibility of an experimental approach to the causality of the association: If homocysteine-lowering prevents thrombosis, hyperhomocysteinemia is likely to indeed be causally linked to thrombosis. This would of course be of much wider relevance than just for etiology, and be of great interest to patients and clinicians since homocysteine is a prevalent risk factor and such therapy, if efficacious, has been estimated to be able to

prevent as much as 25% of events. Furthermore, vitamin therapy is probably safe.
The experimental approach was a randomized and placebo-controlled secondary prevention trial with high-dose B-vitamins in patients with a primary, idiopathic venous thrombosis and hyperhomocysteinemia (chapter 2 and 8).

Two studies in this thesis deal with laboratory aspects of plasma homocysteine. Blood collection for determination of the homocysteine concentration should be performed meticulously and stored at 0°C before separation of the plasma to prevent ongoing synthesis of homocysteine ex vivo[18]. Since this was not feasible in the setting of a multicentre trial, we sought a blood collection tube which stabilizes the blood at room temperature. This led to two investigations: first, we tested the stability of homocysteine in acidic citrated tubes (chapter 3). In previous (unpublished) studies it was made likely that acidic citrate might have this stabilizing effect. The second study was based on the results of the first study where we found a difference in baseline homocysteine concentrations measured in acidic citrate plasma in comparison to EDTA plasma. Measurement of homocysteine in EDTA plasma is considered to be the gold standard. We therefore compared the measurement of homocysteine in acidic citrate with the measurement in EDTA more extensively and used different measurement methods to explore differences between these methods (chapter 4).
The study described in chapter 5 deals with the effect of oral anticoagulants on homocysteine concentrations. Many studies in venous thrombosis have been performed with patients who were treated with anticoagulant therapy. If coumarin derivates have an effect on homocysteine concentrations, the results of those studies may have been flawed. Such an observation might therefore be relevant for an adequate interpretation of homocysteine values for patients using anticoagulant drugs both in epidemiological studies as well as for individual risk estimation. We performed a follow-up study with patients who were going to be treated with anticoagulants after orthopedic surgery and measured homocysteine concentrations before, during and after the treatment period. We also estimated the potential error in previous studies that included such patients. In addition we studied the influence of anticoagulants on homocysteine levels in a group of healthy individuals. (chapter 5).
Another aspect of the relation between homocysteine and venous thrombosis is the pathophysiological mechanism by which hyperhomocysteinemia leads to the development of a thrombus. Several mechanisms have been proposed in the literature[19-28]. We investigated the influence of homocysteine on the coagulation pathway by means of the endogenous thrombin potential (ETP). The ETP reflects the capacity to generate thrombin and is elevated in patients with prothrombotic conditions, such as factor V Leiden, antithrombin deficiency

and oral contraceptive use[29,30]. We compared the endogenous thrombin potential in patients with hyperhomocysteinemia and normohomocysteinemia (chapter 6).

During the screening of patients for the intervention trial (chapter 8) we noticed high homocysteine concentrations in elderly patients. Homocysteine concentrations increase exponentially with increasing age[31], and so does the incidence of thrombosis[32]. Little is known about the risk of thrombosis of elderly people with hyperhomocysteinemia. If hyperhomocysteinemia is a cause of thrombosis, homocysteine-lowering therapy could have a great effect, especially in this age group where hyperhomocysteinemia and thrombosis are frequent. We designed a case-control study including elderly patients with an idiopathic thrombosis and healthy controls, selected from general practices (chapter 7).

In the general discussion (chapter 9) we summarize our studies, with emphasis on the therapeutic options in hyperhomocysteinemia and venous thrombosis.

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Chapter 2

Homocysteine and venous thrombosis: outline of a vitamin intervention trial

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Abstract

In the past years several case-control studies established the association of an elevated plasma homocysteine concentration and the risk of venous thromboembolism. It is still unclear if elevated homocysteine concentrations can cause venous thrombosis. The VITRO (VItamins and ThROmbosis) trial is the first multicenter, randomized, doubleblind and placebo-controlled study to evaluate the effect of homocysteine-lowering therapy by means of 5 mg folic acid, 0.4 mg vitamin B12 and 50 mg vitamin B6. The study is a secondary prevention trial in 600 patients who suffered from a first episode of idiopathic deep vein thrombosis (DVT) or pulmonary embolism (PE), or both. There will be 300 hyperhomocysteinemic and 300 normohomocysteinemic patients included, all with an objectivated venous thrombosis. The end point is recurrence of venous thrombosis.

Introduction

Venous thromboembolism is a common illness with an incidence of 1 to 2 per 1000 per year[1,2]. Common causes of venous thromboembolism are acquired factors (cancer, immobility, fractures of the leg, knee or hip operations and use of oral contraceptives) or hereditary factors (deficiencies of protein C, protein S, and antithrombin[3], high levels of Factor VIII[4], mutations in the Factor II gene[5] and in the Factor V gene resulting in activated protein C resistance[6]). In the past two decades much emphasis has been laid on the role of mild hyperhomocysteinemia as a possible risk factor of venous as well as arterial thromboembolism. In this article we will discuss the association of hyperhomocysteinemia and venous thrombosis, and we will give the design of the VITRO study, a secondary prevention study on the effect of homocysteine-lowering therapy on recurrence of venous thrombosis.

Homocysteine and venous thrombosis

Given the high incidence of arterial thrombotic disease in patients with classic homocystinuria, Wilcken and Wilcken[7] investigated the association of homocysteine and arterial thrombotic disease in patients with premature arterial disease. The association they found was later confirmed by a number of retrospective and prospective studies[8]. These studies established hyperhomocysteinemia as a possible risk factor for arterial thrombotic disease. Hwever, patients with homocystinuria are afflicted not only by arterial thrombosis but also by venous thromboembolism (VTE). By analogy to the hypothesis of Wilcken and Wilcken[7] in arterial disease, several studies have been performed since 1991 to investigate the association of homocysteine and venous thrombotic disease. The epidemiological evidence of homocysteine as a risk factor for venous thrombosis, however, is not as abundant as for arterial thrombotic disease (Table 2.1).

Table 2.1 Published studies on the relation of homocysteine and venous thrombosis.

Authors	Publication Year	Study Method	Age	Cut-Off tHcy	Fasting/ MLT[a]	Cases (N)	Controls (N)	Elevated tHcy Cases (N)	Elevated tHcy Controls (N)	Odds Ratio (95% CI)
Brattstrom et al.[9]	1991	case-control	<50	mean +2 SD	fasting	42	42	4	3	1.4 (0.3–6.5)[b]
					MLT			6	2	3.3 (0.6–17.6)[b]
Bienvenu et al.[10]	1993	case-control	<57	mean +2.7 SD	Fasting[c]	23[f]	49	7	0	—
Falcon et al.[11]	1994	case-control	<50	mean +2 SD	fasting	80	51	7	0	—
					MLT	79	40	14	1	8.4 (1.1–66.4)[b]
Amundsen et al.[14]	1995	case-control	<57	mean +2 SD	fasting	35	39	2	1	2.3 (0.2–26.6)[b]
					MLT			2	1	2.3 (0.2–26.6)[b]
Fermo et al.[15]	1995	case-control	mean 36	95th percentile	fasting	107	60	10	3	2.0 (0.5–7.4)[b]
					MLT	58	60	11	3	3.7 (1.0–13.8)[b]
den Heijer et al.[12]	1995	case-control	<88	90th percentile	fasting	185	220	46	21	3.1 (1.8–5.5)
					MLT			44	20	3.1 (1.7–5.5)
Cattaneo et al.[16]	1996	case-control	?	95th percentile	fasting	89	89	7	4	1.8 (0.5–6.4)
					MLT			7	4	1.8 (0.5–6.4)
den Heijer et al.[13]	1996	population based case-control	<70	95th percentile	fasting[c]	269	269	28	13	2.5 (1.2–5.2)
Simioni et al.[17]	1996	case-control	<92	90th percentile[d]	fasting	60	148	15	17	2.6 (1.1–5.9)
Ridker et al.[18]	1997	prospective, nested case-control	mean 60	95th percentile	fasting[c]	145	646	10	29	1.6 (0.8–3.3)
						?	?	?	?	3.4 (1.6–7.3)[e]
Eichinger et al.[19]	1998	prospective	<85	95th percentile[d]	fasting	28	236	12	54	2.7 (1.3–5.8)

[a] MLT, methionine loading test: post-methionine loading tHcy or increase of tHcy compared with baseline after loading; [b] odds ratio calculated from the published data; [c] non-fasting; [d] from a previously selected reference group; [e] only idiopathic thrombosis are analyzed; number of patients is not published; [f] venous and arterial thrombosis.

Retrospective Studies

In 1991 Brattstrom et al.[9] published a study in which average homocysteine concentrations did not differ between patients with VTE and controls. Patients, however, had an elevated level more often, although it was not significant because of low sample size. Bienvenu et al.[10] in 1993 found a distinct difference in homocysteine concentrations between patients with either arterial or venous thrombosis and healthy controls. This finding was confirmed by Falcon et al.[11] Our group published a study in 1995: fasting and post-methionine homocysteine concentrations of 185 patients with a history of recurrent VTE and 220 controls from a general practice were compared[12]. Odds ratios of 3.1 were found for both preload and postload homocysteine concentrations above the 90th percentile of the control group (18.6 µmol/l). An increase of the risk was already seen at homocysteine concentrations of 14.0 µmol/l. A second study was performed to estimate the risk of homocysteine for a first episode of VTE. Homocysteine concentrations of a subgroup of patients participating in the Leiden Thrombophilia Study were analyzed[13]. All patients had had a first, objectively confirmed DVT. Baseline but no postload homocysteine levels were available. An odds ratio of 2.5 was calculated for homocysteine concentrations above the 95th percentile of the control group. Also, in this group the odds ratios increased with higher cut-off concentrations. The effect was independent from known hereditary risk factors for VTE such as protein C, protein S, or antithrombin deficiency and, unexplainably, more pronounced in women than in men. No interrelation between Factor V Leiden and hyperhomocysteinemia could be established as a result of the small number of subjects with both abnormilities.
More case-control studies were published subsequently that confirmed the association of hyperhomocysteinemia and venous thrombosis[14-17].

Prospective Studies

Only two prospective studies have been published so far. The U.S. Physicians Health Study is a prospective cohort study in male physicians[18]. Homocysteine concentrations were determined in 14,916 men at baseline. From these men 158 developed a venous thrombosis during the subsequent follow-up years (mean, 12 years). These cases were matched with 646 controls from the same cohort. When analyzing all VTE cases with controls, the investigators found no association between an elevated homocysteine concentration and VTE. However, when only the cases who had suffered an idiopathic VTE were analyzed, they found a relative risk of 3.4. Furthermore, this study demonstrated an even more increased risk of VTE when both hyperhomo-

cysteinemia and Factor V Leiden were present, suggesting a synergy. Also, this finding was more pronounced when only idiopathic cases of VTE were analyzed.

A second prospective study was performed by Eichinger et al.[19] They selected patients with a first episode of idiopathic VTE. After the event total homocysteine (tHcy) was measured. Patients were followed for recurrence of VTE. Hyperhomocysteinemic patients were found to be at greater risk for recurrence than were the normohomocysteinemic patients. This resulted in a relative risk of 2.6 when corrected for age, sex, and the presence of Factor V Leiden.

In a meta-analysis of 10 published case-control studies on the risk of hyperhomocysteinemia and venous thrombosis, pooled estimates of the odds ratios were calculated[20]. The authors found odds ratios of 2.5 (95% confidence interval (CI) 1.8 to 3.5) for fasting levels and 2.6 (95% CI 1.6 to 4.4) for postmethionine increased concentrations, supporting the hypothesis that hyperhomocysteinemia is a risk factor for venous thromboembolism.

As yet there is no clear evidence how hyperhomocysteinemia could lead to venous thrombosis. How hyperhomocysteinemia might cause thrombosis is subject of a different article in this issue.

Therapy of hyperhomocysteinemia

Folic acid, hydroxycobalamin (vitamin B12), and pyridoxine (vitamin B6) form the key elements in the therapy of hyperhomocysteinemia. These vitamins function as cosubstrate or as cofactor in the metabolic pathways of homocysteine. High-dose pyridoxine, which as pyridoxal-‘5-phosphate acts as cofactor with cystathionine beta-synthase (CS) in the transsulphuration pathway, is the vitamin that is used in the treatment of classical homocysteinuria. Therapy with pyridoxine is suggested to decrease the incidence of vascular complications in these patients[21,22]. A meta-analysis of 10 studies by the Homocysteine Trialists’ Collaborators showed that in mild hyperhomocysteinemia folic acid is the main homocysteinelowering vitamin[23]: 0.5 to 5 mg of folic acid daily reduced homocysteine concentrations by about 25%. An extra 7% decrease was produced by 0.5 mg/d of vitamin B12. Not only elevated levels of homocysteine but also concentrations usually regarded as normal (<16 µmol/l) were decreased by vitamin supplementation[24]. Similar decreases were found in patients with venous thrombosis as in controls[24].

VITRO study

Study rationale

The clinical key question is whether a decrease of homocysteine concentrations will prevent thromboembolic events[25].This is a question that needs to be answered: It will make us understand more about homocysteine as a possible causal agent of thromboembolic disorders, and it could have great impact on the prevention of vascular diseases[8]. (Although homocysteine is not a strong risk factor, it is, when defined as levels above the 75th or 90th percentile, a very prevalent one.)

In order to answer this question, intervention studies are needed. These studies should be randomized and placebo controlled and therefore not susceptible to bias or confounding factors such as vitamin supplementation or changing dietary habits that can easily influence homocysteine levels. Some investigators argue the need for randomized trials because a substantial amount of evidence is available from retrospective and prospective studies and vitamin therapy appears to be safe. However, every therapeutic intervention deserves a proper evaluation before it is used on a broad scale. Furthermore, although vitamin therapy appears to be safe, the risk of unsuspected adverse effects should be taken into account in every intervention.

In 1996 the VITRO trial was started in the Netherlands. The VITRO trial is a study on the effect of vitamin B in the secondary prevention of venous thrombosis. It is a multicenter, randomized, double-blind and placebocontrolled trial. The study is a collaborative project of the Department of Hematology of the Leyenburg Hospital in The Hague, the Department of Clinical Epidemiology of the Leiden University Hospital, the Laboratory of Pediatrics and Neurology of the Nijmegen University Hospital, and the anticoagulation clinics of The Hague, Rotterdam, Amsterdam, Utrecht, Leiden, Amersfoort, and Delft, The Netherlands. Furthermore, there are four participating centers outside the Netherlands: in Vienna, Austria, and in Milano, Bolzano, and Bologna, Italy.

Study objective

The trial was designed to evaluate whether the effect of homocysteine-lowering therapy by means of multivitamin B in patients with a primary venous thromboembolism and hyperhomocysteinemia leads to a reduction of recurrent thrombosis.

Patient selection

In the Netherlands almost all patients treated with oral anticoagulants are registered at anticoagulation clinics after hospital discharge or directly after

diagnosis when treated in an outpatient setting. The anticoagulation clinics monitor the treatment by international normalized ratio (INR) measurements and adjust the dose of the coumarins. Patients are recruited for the VITRO study by means of these anticoagulation clinics (Figure 2.1).

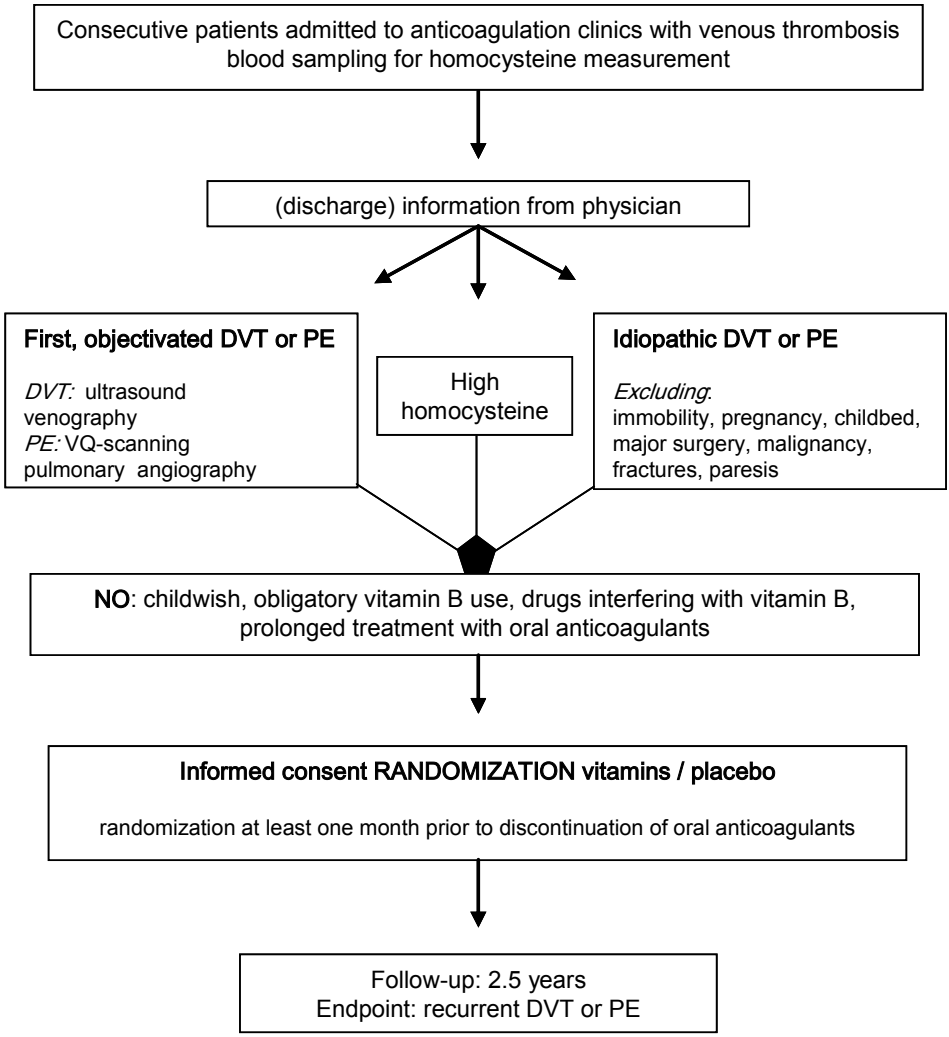

Figure 2.1 VITRO trial: patient selection and randomization.

All new patients registered with the diagnosis of primary DVT or PE are asked to donate an extra blood sample for measurement of their homocysteine concentration. The previously mentioned centers cover a well-defined geographical area of about 3.5 million people in the Netherlands. Because these centers treat nearly all patients with venous thrombosis, the VITRO study is able to include patients without substantial referral bias. The blood is sampled in tubes containing acidic citrate as anticoagulant. In these tubes the blood is stabilized at room temperature for measurement of the homocysteine concentrations[26]. Homocysteine values measured in these tubes correlate very well with those measured in classically used tubes, in other words, tubes containing ethylenediaminetetracetic acid (EDTA) as anticoagulant, that are put on melting ice immediately after blood collection until processing (Willems et al., unpublished data). The blood is processed in the anticoagulation clinic and the plasma is sent to Nijmegen for determination of the homocysteine by regular mail because plasma samples stay stable for several days at room temperature[27]. In the meantime, information is retrieved from the patients' general practitioner or specialist about the diagnosis and circumstances in which the patients developed their thrombosis. Once patients have met all entry criteria, they are asked to participate in the study 1 month before the treatment with the coumarins is terminated. In this month a substantial homocysteine decrease can be achieved[23]. Patients have to give their informed consent in accordance with the current revision of the declaration of Helsinki (1975) in order to participate in the study.

Hyperhomocysteinemic and normohomocysteinemic groups

Two groups of patients are recruited for the VITRO study: a group of patients with hyperhomocysteinemia and a group of similar size with patients with normohomocysteinemia. Because the number of patients with normohomocysteinemia presented by the anticoagulation clinics is greater than the number with hyperhomocysteinemia, a random selection of all normohomocysteinemic patients is made in order to achieve a parallel inclusion: one normohomocysteinemic patient for every hyperhomocysteinemic patient included.

Entry criteria

1. Objectively confirmed, primary, proximal DVT (diagnosed with compression ultrasonography or venography) or PE (diagnosed with "high probability" ventilation-perfusion [V/Q] scanning or pulmonary angiography), or both
2. Homocysteine concentrations above the 75th percentile of a reference group of the general population from which the patients are selected (hyperhomocysteinemic group) or homocysteine concentrations below the 75th percentile of this reference group (normohomocysteinemic group)

3. Age 20 to 80 years
4. Absence of other major risk factors for DVT such as major abdominal surgery, major hip and knee operations, fractures of the legs or hips, multitrauma, malignant disease, pregnancy and childbirth, paralysis of the legs, immobility for more than 3 weeks
5. Ability to start the study at least 4 weeks before discontinuation of the coumarins in order to ensure substantial homocysteine decrease at that time Patients with the following conditions are excluded: (obligatory) use of vitamin B, continued use of coumarins, pregnancy or trying to get pregnant, and the use of medications that are influenced by folic acid (phenytoin, L-dopa, methotrexate).

Randomization

Randomization is done by someone not involved in the treatment of the patients. The randomization is stratified by gender and by anticoagulation clinic. Randomization tables with six and four random permuted blocks are used for the randomization.

Treatment schedule and trial medication

The treatment group will be treated with 1 capsule daily containing 5 mg folic acid, 400 μg hydroxycobalamin, and 50 mg pyridoxine. The stability of this combination has been tested and proven valid for the duration of the study. The placebo group will take 1 placebo capsule daily. The study medication is provided by Dagra Farma, Amsterdam, The Netherlands.

Follow-up

Randomized patients are seen at outpatient clinics at the start of the study and 3, 6, and 24 months after randomization. Because the time span between the follow up visits to the outpatient clinics is quite large, patients will be interviewed by telephone every 6 months. The duration of the study for each patient is 2.5 years. Because the last visit to the outpatient clinic is already after 2 years, the trial will end for each patient after an interview
by telephone. At each follow-up visit blood is sampled for determination of tHcy. By means of determination of tHcy, therapy compliance is measured. Patients are seen after overnight fasting. Patients receive their study medication at these follow-up visits, and the leftover capsules will be taken back.

Ultrasonographies

In patients with DVT of the leg, a compression ultrasonography (cUS) is performed 3 months after the thrombosis. The cUS is done to enhance the diagnosis of a possible recurrence. If residue of the old thrombus is seen on the cUS, the cUS is repeated 6 and, if necessary, 12 months after the thrombosis. In patients with a PE, a cUS of both legs is performed to exclude a DVT. The ultrasonographies are performed in one hospital or institution in every participating city. The results of the cUS are noted in a so-called patient passport, a booklet that patients are instructed to give to the physician if signs and symptoms of a thrombosis recur. By using this passport, the data on the residual thrombosis are available, even if patients visit other hospitals. The diagnosis “recurrent DVT” can be made when a previously normal or normalized venous segment cannot be compressed or when there is an increase in the diameter of residual thrombus by 4 mm[28,29].

Endpoint

Endpoint in the study is recurrent DVT or recurrent PE, or both, as diagnosed by the treating physician and scored definitive if anticoagulants are prescibed. The diagnosis might be confirmed by using objective tests as described before. Analysis will be done on the confirmed and nonconfirmed (but indicating clinical relevance) recurrent events and on the confirmed recurrences only.

Study size

The study size is calculated for the hyperhomocysteinemic group. With α=0.05 and β=0.2 and with a recurrence percentage of 20% in the placebo group and a 50% risk reduction because of the vitamin therapy, 155 patients in each treatment group are required if tested one sided. This means that more than 310 patients will be randomized. A similar number of patients will be included in the normohomocysteinemic group, resulting in a total study size of 620 patients.

Possible outcome and outlook

For the VITRO study only patients with an idiopathic venous thrombosis are selected. It is hypothesized that these patients have no apparent cause of their thrombosis; therefore, the contribution of homocysteine in the etiology of the thrombosis might be greater than in patients with very apparent causes such as bedrest, hip fractures, and so on. This hypothesis was later confirmed by the results of the study by Ridker et al [18], which showed a higher odds ratio in idiopathic thrombosis than it did in nonidiopathic thrombosis. A second

assumption is that patients with idiopathic DVT or PE are at higher risk for recurrent thrombosis in comparison with patients with an environmental and reversible cause [30]. Based on the results of the case-control studies, homocysteine is a risk factor for venous thrombosis. However, it is not a certainty that lowering homocysteine concentrations will have an effect on recurrence of events. Most case-control studies are retrospective and do not address the issue of causality; they only show associations. It could be hypothesized that homocysteine is a result of the thrombosis, in which case homocysteinelowering therapy will not result in a decrease in the number of recurrent events. However, from the results of the two prospective studies, this is not likely. Another, more likely assumption is that the homocysteine increase is an epiphenomenon: an unknown disorder leading to thrombosis as well as to homocysteine increment. If homocysteine is such an epiphenomenon, prospective case-control studies will show an association, but homocysteine-lowering therapy will not take away the causal agent of the thrombosis and intervention will not result in a decease of recurrent events. Even if homocysteine is a causal agent in the development of venous thrombosis, the effect of homocysteine- lowering therapy on *recurrent* thrombosis can only be assumed. Only two of the previously discussed studies address the association of homocysteine and recurrent thrombosis. The study by den Heijer *et al*. [12] on recurrent thrombosis shows an odds ratio that is very much in the range of the odds ratios found in studies on primary thrombosis. One could conclude that homocysteine is a risk factor only for primary thrombosis and not a risk factor for recurrence any more when patients have already had their first thrombotic event. Other risk factors, for instance an insufficient venous system caused by the first thrombosis, then play a much more important role that diminishes a possible effect of homocysteine.

Lowering homocysteine concentrations would not make a great difference or would even be useless. The study by Eichinger *et al*.[19] however, estimated the risk of recurrent thrombosis compared with patients who already had a first thrombotic event and showed an odds ratio of 2.7, supporting the hypothesis that homocysteine-lowering therapy could have an effect on recurrent thrombosis.

In this study two groups of patients were selected, a hyperhomocysteinemic and a normohomocysteinemic group. Based on the results of case-control studies, the risk of disease in this latter group is lower than it is in the hyperhomocysteinemic group [12,13,19].The addition of a normohomocysteinemic group can provide us with important extra information. Four types of outcome can be hypothesized.

1. There is no effect of vitamin therapy on recurrence in both groups. This outcome hypothesis is discussed earlier.

2. Vitamin therapy will only have an effect in the hyperhomocysteinemic group, suggesting that hyperhomocysteinemia above a certain concentration is a causal agent in the development of venous thrombosis.
3. Vitamin therapy will have a similar effect in both the hyperhomocysteinemic and the normohomocysteinemic group, suggesting a pathophysiological mechanism that acts independent of homocysteine (e.g., folic acid mediated).
4. The effect of vitamin therapy is greater in the hyperhomocysteinemic group than it is in the normohomocysteinemic group. This suggests a graded association of homocysteine and venous thrombosis: The effect of vitamins will be more pronounced in patients with higher levels of homocysteine. Because vitamin therapy causes homocysteine decrease, even of previous "normal" concentrations[24] effect of vitamins on recurrence of thrombosis in the normohomocysteinemic group cannot be ruled out. Although there are not sufficient data from the case-control studies done in venous thrombosis, studies in arterial vascular disease suggest such a graded response[8]. Based on the results from the hyperhomocysteinemic group, trend analyses will be performed in the VITRO study to measure the effect in the normohomocysteinemic group.

In 1996, the trial was started in The Netherlands. In 1998, the four centers outside The Netherlands were approached for participation and started randomizing patients. These centers are provided with the same study medication and randomize patients according to the same entry criteria. Homocysteine measurements are done in each respective center and the cut-off level of the homocysteine is based on the 75th percentile of the local reference group. By July 1999, more than 500 patients were randomized. The study is expected to be complete in the beginning of 2000. Results of the study can be expected by the end of 2002.

If homocysteine lowering effectively decreases the number of recurrent venous thromboembolic events, this will be of great benefit for patients. It will not only reduce the risk of recurrence but also diminish morbidity and mortality resulting from the use of long-term anticoagulant therapy in patients after a second event. In addition, if vitamin treatment will reduce the number of events it will prove the relevance of homocysteine metabolism as a risk factor in vascular disease, a conclusion impossible to make from case-control studies and still a subject of debate in the prospective studies.

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Chapter 3

Acidic citrate stabilizes blood samples for assay of total homocysteine

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Introduction

Homocysteine is a sulfhydryl-containing amino acid, formed by demethylation of the essential amino acid methionine. Homocysteine is either transsulfurated to cysteine or is remethylated to methionine by methionine synthase. Excess intracellular homocysteine is likely to be transported to the extracellular compartment[1]. Increasing evidence indicates that homocysteine is implicated in the pathogenesis of thromboembolic diseases. Several case control studies have shown a relationship between increased total plasma homocysteine (tHcy) concentrations and an increased risk of arterial[2-4] and venous thrombosis[5–8]. An increase of the tHcy concentration of 5 µmol/l is associated with 1.5–1.9 times increased risk for coronary artery or cerebrovascular disease[9].These values indicate that small differences may be of clinical importance. Therefore, practical standardized conditions for handling blood specimens for tHcy determination are required. In most studies, blood is drawn in tubes containing K3EDTA. The whole-blood sample is immediately put on crushed ice and then centrifuged as soon as possible to prevent an increase of tHcy concentrations. This tHcy increase is caused by ongoing homocysteine metabolism in blood cells, the majority of which are red blood cells[10,11]. This blood handling procedure is not practical, particularly when larger studies are conducted outside a hospital setting; even in a routine clinical setting, this protocol might be hard to put into practice. To find an alternative, more suitable blood-collection medium, we investigated the effect of different blood-collection media on tHcy production when whole blood is kept at room temperature for 6 hours.

Methods

Blood was drawn by venipuncture of the antecubital vein from laboratory coworkers or from consecutive patients who visited the outpatient clinics of the Leyenburg Hospital in The Hague for various reasons, unknown to the authors. Informed consent was obtained in accordance with the current revision of the Helsinki declaration of 1975. Two studies were performed. A pilot study was done with blood from 11 patients and 11 laboratory coworkers (12 men and 10 women; ages 18–63 years). Blood was drawn in tubes with 1.8 g/l K3EDTA (Vacutainer Tube; Becton Dickinson), in tubes with 2.5 g/l sodium fluoride and 2 g/l potassium oxalate as anticoagulant (Vacutainer Tube), in tubes with 0.5 mol/l acidic citrate (Biopool StabilyteTM), and in tubes with a mixture of the sodium fluoride, potassium oxalate, and acidic citrate. Care was taken that all tubes were completely filled by blood. The EDTA-treated blood was put on crushed ice immediately after venipuncture. The other blood samples were

kept at room temperature. The results of this pilot study indicated that tHcy concentrations remained stable in acidic citrate. We conducted a second study to explore this phenomenon more extensively. This main study was done in 30 laboratory coworkers (17 men and 13 women; ages 18–52). Blood was taken in tubes with EDTA and acidic citrate (as described above). From every volunteer, one-half volumes of the tubes containing EDTA were kept at room temperature; the other half were put on crushed ice immediately after sampling. From the tubes with acidic citrate, one-half volumes were kept at room temperature, and the other half were stored in water of 37°C. In both study groups, the blood was centrifuged for 10 min at 2000*g* as soon as possible (within 15 min) after sampling ("0 h") and 2, 4, and 6 h after the venipuncture.

After separation, the plasma was stored at -20°C until determination of the tHCy concentration at the Laboratory of Pediatrics and Neurology of the University Hospital Nijmegen (by H.J.B. and S.V.) by automated HPLC with reversed-phase separation and fluorescent detection [Gilson 232–401 sample processor (Gilson Medical Electronics, Inc.), Spectra-Physics 8800 solvent delivery system, and Spectra-Physics LC 304 fluorometer], according to the method described by Fiskerstrand *et al.*[12] with some modifications[13]. The tHcy concentrations from the tubes containing the acidic citrate were corrected for the dilution caused by the fluid already present in the tube before blood collection.

Paired-sample *t*-tests were used to calculate the significance of the increase of tHcy concentrations in the collection media. Paired-sample *t*-tests were also used to calculate the significance of the differences between the tHcy concentrations in the different collection media at baseline. Results of the *t*-tests are given as the intervals that show $P<0.05$ significance [95% confidence intervals (CI)].

Results

Results of the pilot study are shown in Table 3.1. tHcy concentrations in blood taken in tubes containing sodium fluoride rose markedly after 2 h (0.9 µmol/l; 95% CI 0.5 to 1.3 µmol/l). In the tubes containing sodium fluoride with acidic citrate, tHcy concentrations remained stable for 4 h. After 6 h, there was a slight increase of 0.6 µmol/l (95% CI 0.0 to 1.1 µmol/l). tHcy concentrations in blood taken in tubes containing EDTA that were stored at 0°C and tHcy concentrations taken in tubes with acidic citrate that were kept at 21°C did not rise markedly for 6 h [0.0 µmol/l (95% CI -0.5 to 0.6 µmol/l) and 0.4 µmol/l (95% CI -0.5 to 0.9 µmol/l), respectively]. Results of the main study are shown in Figure 3.1 and in Table 3.1. tHcy concentrations in the EDTA-containing tubes that were put on ice did not rise markedly for 6 h (0.3 µmol/l; 95% CI -0.1 to

0.7 µmol/l). tHcy concentrations in the EDTA-containing tubes that were stored at room temperature rose 2.0 µmol/l after 2 h (95% CI 1.6 to 2.4 µmol/l) and up to 4.7 µmol/l (95% CI 4.1 to 5.3 µmol/l) after 6 h. At room temperature, the tHcy concentrations in acidic citrate did not rise markedly for 6 h after collection (0.3 µmol/l; 95% CI -0.2 to 0.7 µmol/l), whereas tHcy concentrations in blood collected in acidic citrate stored at 37°C increased markedly after 4 h (0.9 µmol/l; 95% CI 0.5 to 1.3 µmol/l).

We found a difference of 1.3 µmol/l (95% CI 0.9 to 1.6 µmol/l) between tHcy concentrations measured in blood sampled in tubes with EDTA and stored at 0°C and tHcy concentrations measured in blood sampled in tubes with acidic citrate kept at room temperature. Such significant differences were present at all measurement times. When the results of the pilot study and the main study were combined, this difference decreased but was still significant (0.8 µmol/l; 95% CI 0.4–1.1 µmol/l; range, -3.1 to 2.2 µmol/l).

Table 3.1 Mean (±SD) increase in homocysteine (in µmol/l) in whole blood in different collection media.

		Pilot study (n=22)				Main study (n=30)			
			Increase after				Increase after		
Storage medium	Temp. °C	Baseline	2 h	4 h	6 h	Baseline	2 h	4 h	6 h
EDTA	0	12.5 (2.4)	0.1 (1.1)	0.4 (0.9)	0.1 (1.2)	12.7 (3.3)	0.1 (1.3)	0.1 (1.3)	0.3 (1.1)
Acidic citrate	21	12.6 (2.6)	0.1 (1.1)	0.4 (0.8)	0.4 (1.1)	14.0 (3.6)	0.0 (1.1)	0.0 (1.1)	0.3 (1.2)
NaF	21	11.3 (1.8)	0.9 (0.9)	1.6 (0.8)	1.7 (1.3)				
Acidic citrate and NaF	21	11.6 (1.9)	0.1 (0.9)	-0.1 (1.0)	0.6 (1.2)				
EDTA	21					13.3 (3.4)	2.0 (1.0)	3.5 (1.4)	4.7 (1.7)
Acidic citrate	37					13.9 (3.5)	0.0 (1.0)	0.9 (1.1)	1.2 (1.5)

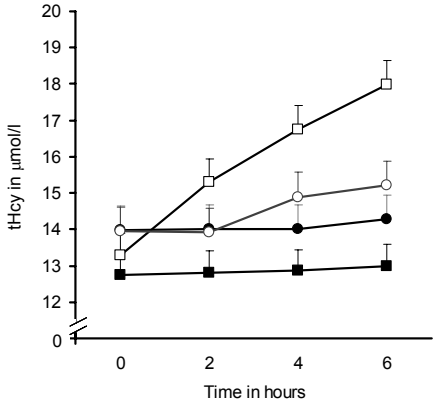

Figure 3.1 Increase of mean tHcy concentrations in whole blood of 30 laboratory coworkers processed 0, 2, 4, and 6 h after collection. Blood was collected in tubes containing EDTA and stored at 0°C (■) or at room temperature (□), and blood was collected in tubes with acidic citrate and stored at room temperature (●) or stored at 37°C (○); bars, SEM of tHcy concentrations.

Discussion

Blood cells produce homocysteine, which can lead to falsely increased plasma homocysteine concentrations. Thus, in the ideal setting, blood cells should be separated from plasma immediately after collection. In EDTA- containing blood, we found that tHcy concentrations increased at room temperature, which has been demonstrated before[10,14]. Even storage at 4°C has been associated with a smaller but steady increase of the tHcy concentration[10]. However, we found that tHcy concentrations remained stable for 6 h when EDTA-containing blood was stored at 0°C, which confirms the findings of Kittner *et al.*[15].

In a study by Møller and Rasmussen[16], heparin containing tubes with sodium fluoride added to a concentration of 2 or 4 g/l of blood prevented the increase of tHcy for only 2 h. In our pilot study and also in the study by Ubbink *et al.*[10], comparable results of increasing tHcy concentrations were found by using 2.5 g/l sodium fluoride.

This investigation is the first concerning the stability of tHcy concentrations in whole blood containing acidic citrate. This tube contains citrate at a low pH of 4.3 (pH±5.9 after blood collection) and was originally developed for the determination of fibrinolytic markers. During studies on fibrinolytic markers in relation to homocysteine, we found that tHcy might not increase in these samples at room temperature (data not shown). In our pilot study, we tested the combination of acidic citrate with sodium fluoride but found no difference between the tubes containing just acidic citrate and the tubes combining acidic citrate with sodium fluoride. Therefore, the stability of tHcy could be solely attributed to the acidic citrate fluid, making the addition of sodium fluoride unnecessary.

We performed this study to find a collection medium that is suitable in epidemiological field conditions. We therefore also tested how tHcy concentrations behave when whole blood, taken in acidic citrate tubes, is stored at a temperature higher than room temperature. When blood was stored at 37°C, tHcy concentrations were increased after 4 h. We do not know the mechanism by which the acidic citrate keeps tHcy concentrations stable. It is possible that the enzymes involved in homocysteine metabolism are blocked at this low pH. This blockage, however, is temperature dependent because tHcy concentrations rise when blood is stored at 37°C. Baseline tHcy concentrations in the EDTA-containing tubes kept at room temperature and centrifuged at t=0 h were higher than the concentrations in EDTA tubes put on ice and centrifuged at 0 h. As mentioned earlier, there was a time interval between the venipuncture and the centrifugation of the blood at t=0 of, at most, 15 min. When cooling of the blood instantly stops the export of homocysteine, tHcy concentrations increase in blood that is kept at room temperature from the time between the blood samples being taken and the separation of plasma from

blood cells because of ongoing export, explaining the difference at t=0. We also found a difference between the mean baseline concentrations of tHcy measured in EDTA- treated blood that was stored at 0°C and tHcy concentrations in blood taken in acidic citrate. This difference can only partly be explained in the same way as above (i.e., a rise of tHcy concentrations in the time interval between blood sampling and separation). Other factors, such as a higher osmolarity of EDTA than acidic citrate, leading to higher plasma volumes, could be responsible for the difference. This, however, needs needs further investigation. This study was conducted to find an alternative collection medium for the determination of tHcy concentrations in plasma. When blood is collected outside a hospital or laboratory setting (e.g., in a large epidemiological study), it is not always possible to put blood tubes on crushed ice. Even within hospitals, it can easily be forgotten or found impractical to put the tubes on crushed ice before processing. Therefore, this method can be prone to inappropriate handling of blood for determination of tHcy concentrations. This study shows that acidic citrate is a good alternative when screening patients in an epidemiological field study because tHcy concentrations stay stable for 6 h. However, because of the differences found between the mean baseline tHcy concentrations in EDTA on ice and in acidic citrate at room temperature, new reference values need to be established before tHcy concentrations obtained in EDTA tubes and stored on crushed ice can be replaced by acidic citrate tubes for determination of tHcy concentrations in individual patients.

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Chapter 4

Measurement of total homocysteine concentrations in acidic citrate- and EDTA-containing tubes by different methods

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Abstract

In epidemiological studies, blood handeling for measurement of homocysteine is cumbersome because at room temperature homocysteine production in whole blood continues after blood collection. Acidic citrate stabilizes homocysteineproduction in whole blood at room temperature. In a previous study baseline differences in homocysteine concentration were found between EDTA and acidic citrate anticoagulated blood. This study was performed to further explore this difference in homocysteine concentration.

Blood from 208 volunteers was collected in tubes ontaining EDTA and acidic citrate as anticoagulant. The blood was processed within 30 minutes. Homocysteine determination in the plasma was done with 2 HPLC methods [HPLC(a) and (b)] and with an automated FPIA method.

The mean differences in homocysteine between acidic citrate blood and EDTA blood with HPLC(a), HPLC(b) and FPIA were 1.8 µmol/l (95% CI 1.6 to 2.1 µmol/l), -2.8 µmol/l (95% CI –3.1 to -2.5 µmol/l) and 0.1 µmol/l (95% CI 0.0 to 0.3 µmol/l) resp.

With all three measurement methods homocysteine concentrations in acidic citrate blood correlated well with homocysteine concentrations in EDTA blood. Regression analyses showed a slopes and intercept of 1.01 and 1.7 for HPLC(a), 0.75 and 0.7 for HPLC(b) and 0.95 and 0.7 for the FPIA.

We conclude that acidic citrate can be used for measurement of homocysteine with all three measurement methods. However, when using these methods, new reference values need to be established, because there is a difference in individual concentrations in acidic citrate and EDTA blood.

Introduction

In the past decade several studies have shown an association of hyperhomocysteinemia with arterial vascular disease[1-3], venous thrombosis[4], pregnancy complications[5] and Alzheimers disease[6,7]. Blood collection for homocysteine measurement is usually done in tubes containing EDTA as anticoagulant. The tubes have to be put on melting ice immediately and have to be centrifugated within 1 or 2 hours. At room temperature there is an increase in homocysteine in whole blood[8-12]. This procedure with tubes being placed on ice and centrifugated within 1 or 2 hours is cumbersome in a clinical setting, but in particular in large epidemiological studies. Therefore several methods are proposed to stabilize homocysteine in whole blood.
In a previous study we found that citrate with a low pH (pH=4.3, after blood collection ~5.9) stabilizes plasma homocysteine concentrations in whole blood for 6 hours when the blood is stored at room temperature[12]. However, we found a small difference in absolute homocysteine concentration measured in acidic citrate anticoagulated blood compared to EDTA.
The purpose of the current study is to further explore the differences in homocysteine concentrations between EDTA and acidic citrate anticoagulated blood and to investigate whether the differences are the same for different methods for measurement of homocysteine.

Methods

Blood was collected from volunteers who were selected from a general practice in Beverwaard, Rotterdam, the Netherlands. Thousand people were randomly selected from the practice and addressed by mail to participate as controls in a case-control study. A total of 258 participated. We draw additional blood tubes to perform our comparison study. All volunteers gave their informed consent according to the revised Helsinki declaration.
Blood was collected by venipuncture in 10 ml tubes containing EDTA (Vacutainer^{R}, Bencton&Dickinson, U.S.A.), and 5 ml tubes with acidic citrate (Stabilyte^{R}, Biopool, Sweden) as anticoagulant. Care was taken that the acidic citrate tubes were completely filled in order to obtain a blood:anticoagulant ratio of 9:1 in every sample. Blood collected in the EDTA tubes was stored at 0°C (melting ice) immediately after blood sampling until processing. Blood in the tubes with acidic citrate was kept at room temperature. All samples were processed within 30 minutes. They were centrifugated for 10 minutes at 2000 *g*

in a non-cooled centrifuge. The plasma was divided into aliquots and stored at –30°C until determination of the homocysteine.

The homocysteine was measured with three different methods:

1. HPLC(a): Automated high-performance liquid chromatography (HPLC) with reverse phase separation and fluorescent detection (Gilson 232-401 sample processor (Gilson Medical Electronics Inc., Middleton,WI), Spectra-Physics 8800 solvent delivery system and Spectra-Physics LC 304 fluorometer (San Jose,CA)), according to the method described by Fiskerstrand *et al.*[13] with some modifications[14]. The reagents used for the reduction are NaBH4 and DTE. For the derivatization procedure we used ethylmorpholine buffer and monobromobimane.
2. HPLC(b): Automated high-performance liquid chromatography with reversedphase seperation and fluorescence detection according to Araki *et al.*[15] and modified by Ubbink *et al.*[16]. With this method homocysteine is reduced with tri-N-butylphosphine and the derivatization is done with SBD-F in borate buffer.
3. FPIA: A commercially available fluorescence polarization immunoassay (FPIA)(IMx Homocysteine, Abbott Diagnostics)[17]. This method, as the above mentioned HPLC methods, begins with the reduction of homocysteine using DTT. The homocysteine is enzymetically converted to SAH using adenosine and SAH hydrolase. Subsequent steps are adding mouse monoclonal antibodies and a fluoresceinated tracer before the homocysteine measurement. The assay is fully automated and can easily be applied in laboratories who do not have HPLC equipment but do have the means for an FPIA.

Homocysteine concentrations measured in acidic citrate were corrected for the amount of fluid present in the tube prior to the blood collection by multiplying the measured concentration with 10/9. We did not correct for the fluid present in the EDTA tubes prior to collection (i.e., 0.117 ml), since the difference is negliable (±1%). All homocysteine measurements with HPLC(a) were corrected by subtracting 2.4 µmol/l from the measured concentration, according to the results as descibed by de Bree *et al.*[11].

Statistics

The mean homocysteine concentrations in acidic citrate and EDTA were compared by paired-samples T-tests.
We studied the association of homocysteine concentrations in acidic citate plasma and EDTA plasma with liniair regression for HPLC(a), HPLC(b) and FPIA.

Furthermore, to study the differences in individual values we plotted the mean difference of two individual measurements against the difference, according to the method described by Bland and Altman[18]. This method gives information about the spread of the differences between the individual values. We calculated the mean proportional bias (i.e., the mean difference in percentages) and the corresponding 95% confidence intervals (expressed as 'limits of agreement') after log-transformation of the homocysteine concentrations.

Results

Blood was obtained from 258 volunteers. From 50 volunteers there was an insufficient amount of plasma to perform all six analyses. Of 208 persons (79 male, 129 female; age 23-88, median 65), all six measurement were available for the analyses.

Homocysteine concentrations in EDTA and acidic citrate samples with the 3 measurement methods are shown in boxplots in Figure 4.1. With HPLC(a) mean homocysteine measured in acidic citrate was 1.8 µmol/l (95% CI 1.6 to 2.1 µmol/l) higher than in EDTA. Mean homocysteine in the acidic citrate was lower than in the EDTA samples (mean difference -2.8 µmol/l (95% CI 2.5 to 3.1 µmol/l) when measured with HPLC(b). The mean difference in homocysteine concentration was the smallest with FPIA: 0.1 µmol/l (95% CI 0.0 to 0.3 µmol/l).

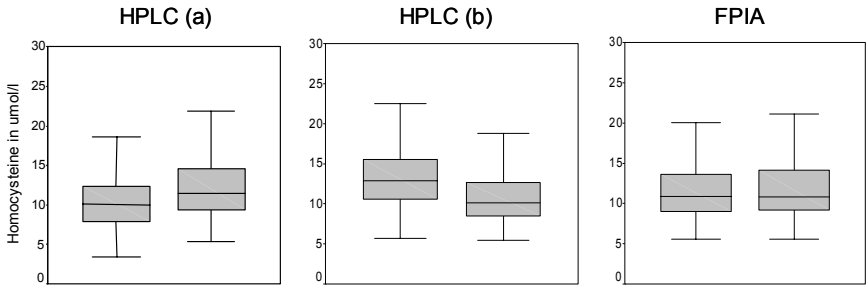

Figure 4.1 Boxplots of homocysteine measured in EDTA vs. acidic citrate anticoagulated blood. Measurement with 2 HPLC methods and FPIA.

The regression analysis of the homocysteine concentrations measured with HPLC(a) shows a slope of 1.01 (95% CI 0.96 to 1.07) and an intercept of 1.7 (95% CI 1.1 to 2.4). With HPLC(b) the slope was 0.75 (95% CI 0.72 to 0.78)

and the intercept at 0.7 (95% CI 0.2 to 1.2), meaning that homocysteine are approximately 25% lower measured in acidic citrate than in EDTA. With FPIA we calculated a slope of 0.95 (95% CI 0.92–0.98) with an intercept of 0.7 (95% CI 0.3 to 1.2). The plots are shown in Figure 4.2a-c.

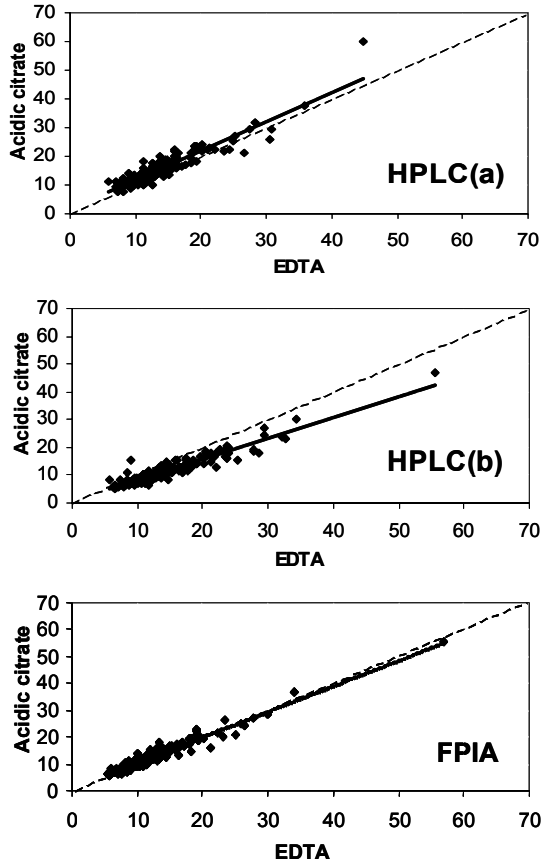

Figure 4.2 Total homocysteine (tHcy) in µmol/l in acidic citrate versus EDTA samples for HPLC(a), HPLC(b) and FPIA. (- - - = line of equality, —— = regression line)

The agreement of the individual concentrations is shown in the Bland-Altman plots. They show the spread of the differences between the individual measurements (Figure 4.3). It shows us that the acidic citrate values agree the most in the FPIA method. This is also shown in Table 4.1 in which the upper and lower levels of agreement are shown after log-transformation. The limits of agreement are the broadest with HPLC(b). This is a result of the fact that the

homocysteine concentrations in the acidic citrate tubes are approximately 75% of the concentrations in EDTA. With increasing concentrations the difference between individual concentrations gets higher but also the mean difference increases, so analysis of the limits of agreement results in a broad interval between the limits of agreement.

Table 4.1 Difference between homocysteine concentrations in acidic citrate and EDTA.

	Mean difference in µmol/l (EDTA – acidic citrate)	95% CI of the mean	Mean proportional bias	Lower limit of agreement	Upper limit of agreement
HPLC(a)	-1.8	-2.1 to -1.6	-16%	-40%	15%
HPLC(b)	2.8	2.5 to 3.1	24%	-4%	62%
FPIA	-0.1	-0.3 to 0.0	2%	-19%	16%

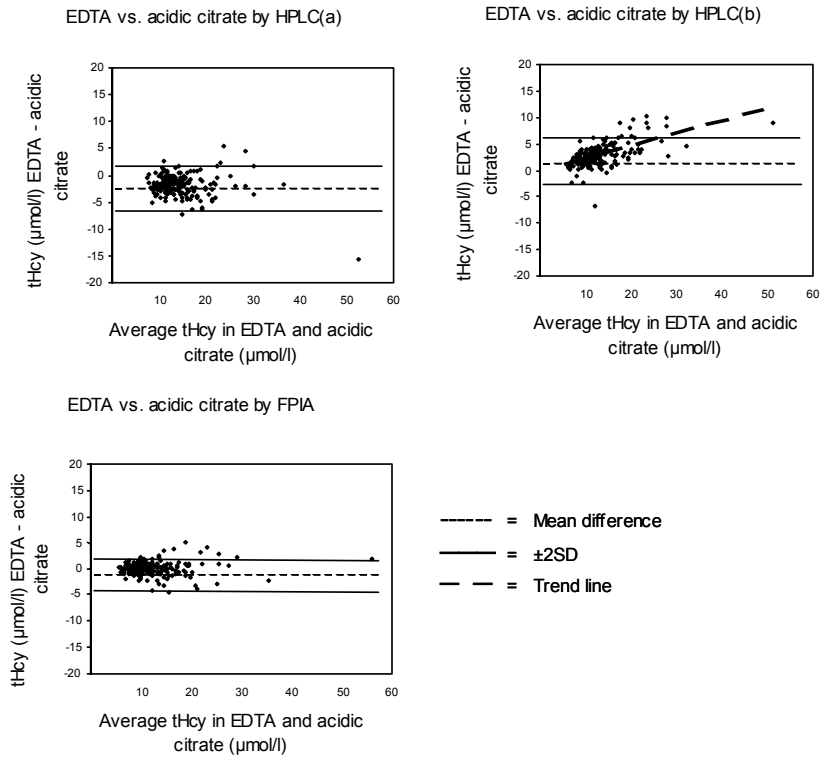

Figure 4.3 Bland-Altman plots of homocysteine measured in EDTA vs. acidic citrate anti-coagulated blood. Measurement with 2 HPLC methods and FPIA.

Discussion

Large epidemiological studies require blood collection techniques that are easily applicable in a field setting. Difficult blood collection techniques are sensitive to blood handling mistakes, which causes loss of information. For homocysteine measurement, storage of whole blood collected in EDTA prior to centrifugation should be done at 0°C to prevent elevation of homocysteine due to the homocysteineproduction by the blood cells, mainly the erythrocytes. Several alternative collection media have been proposed. Fluoride stabilized homocysteine for 2 hours at room temperature in several studies[8,12,19], but this could not be reproduced in other studies[20-23]. Hughes *et al*. also showed that sodium fluoride causes additional dilution defects, leading to lower plasma homocysteine concentrations at baseline and advises not to use NaF as anticoagulant for homocysteine measurement[20]. Probst *et al*. developed a collection device in which whole blood is lysed. Homocysteine remained stable in this lysate for 48 hours at room temperature[24]. Hill investigated 3-deazaadenosine (3DA) and found that EDTA blood with added 3DA stabilized homocysteine for 72 hours when stored at 2-8°C[25]. However, when the blood was stored at room temperature, homocysteine concentrations increased.
We previously reported that acidic citrate stabilizes homocysteine in whole blood at room temperature for 6 hours[12]. Our finding was confirmed by Salazar *et al*.[26]. They found that homocysteine stayed stable up to 6 hours when the measurement was done by HPLC. They used a HPLC method[27] that differed form ours. However, when they measured with FPIA, they found an increase in homocysteine in both acidic citrate and EDTA stored at 0°C[26].

The aim of the current study was to compare homocysteine measurement in acidic citrate plasma with homocysteine concentrations in EDTA plasma. We found that homocysteine measured in blood with acidic citrate as anticoagulant correlates well with homocysteine measured in blood with EDTA as anticoagulant, regardless of the method of measurement used. This is derived from the narrow 95% confidence intervals in the regression analyses. We also found that the absolute individual homocysteine concentrations in both media may differ. The level of agreement was the best with the FPIA method.
We found differences between the mean homocysteine concentrations in acidic citrate compared with EDTA. This difference is not the same for each measurement method, thus depends on the method used. With HPLC(a) the difference is constant, meaning that the homocysteine measured in acidic citrate is approximately 1.7 µmol/l higher than in the EDTA samples. With HPLC(b) the difference is dependant of the level of the homocysteine, the absolute difference rises with increasing concentrations of homocysteine. On

average the homocysteine in the acidic citrate samples is 25% lower than in the EDTA samples. The difference in mean homocysteine was the smallest with the FPIA (0.1 µmol/l), with a regression line close to the line of equality.
The difference we measured with HPLC(a) is comparable to the difference we found previously in a stability study in which homocysteine was 1.3 µmol/l higher in acidic citrate samples[12]. The same HPLC method was used in this study. With HPLC(b) we measured much lower homocysteine in the acidic citate samples. Salazar *et al*. found a smaller difference of 5% between homocysteine in EDTA and acidic citrate samples measured with HPLC, with homocysteine being lower in acidic citrate samples than in the EDTA samples[26]. We have hypothesized about the mechanisms causing homocysteine being higher in acidic citrate than in EDTA samples when measured with HPLC(a), and being lower with HPLC(b). We thought it could be a result of the acidic environement interacting with the measurement method. Therefore we added acid to EDTA plasma and did 10 additional measurements with HPLC(a), but the homocysteine concentrations in the regular EDTA plasma and the acidic EDTA plasma were comparable (data not shown). It may be that the reagents used in the measurement interact with homocysteine from acidic citrated plasma and influence the measurements. Since both HPLC methods use different reagents this could lead to the differences we found.
The difference found between homocysteine in EDTA versus acidic citrate samples with the FPIA was small. In the same study as mentioned above, Salazar also used the same FPIA method as in our study and also found that homocysteine in acidic citrate samples did not differ significantly from the homocysteine concentrations in EDTA samples[26]. Palmer-Toy *et al*. compared homocysteine in EDTA and citrate (i.e., non-acidic citrate) anticoagulated blood measured with FPIA[28]. They also found no difference between homocysteine in the collection media when they corrected the homocysteine values from the citrate samples with a correction factor. This correction factor corrected for hematocrit, based on gender and the added amounts of anticoagulant present in the collection tube before blood sampling. We used the same correction factor to transform our homocysteine values from the acidic citrate samples measured with FPIA and did the regression analysis for the FPIA method again, but this did not influence the results.

Based on the results of this study, we conclude that tubes with acidic citrate can be used in epidemiological studies since homocysteine concentrations correlate highly with those measured in EDTA plasma. Reference values need to be established when using acidic citrate tubes since individual values differ from those measured in EDTA samples and the difference is dependent on the measurement method being used. Individual values differ between EDTA and acidic citrate samples, therefore the two media cannot be compared directly.

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Chapter 5

Oral anticoagulant treatment with coumarin derivatives does not influence plasma homocysteine concentration

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GMJ Bos

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Abstract

Introduction

High circulating levels of homocysteine are a risk factor for arterial and venous thrombosis. This association has been established in numerous case-control studies. In some of these studies patients were treated with anticoagulants at the time of venapuncture. It is not clear whether homocysteine concentrations are influenced by anticoagulants. If there is an effect of anticoagulation on homocysteine levels this might under- or over-score the possible association of homocysteine levels and vascular disease.

Methods

In this study we used two different groups to investigate the association of coumarin derivatives on homocysteine concentrations. Homocysteine levels were measured in patients (N=40) who were on the waiting list for orthopedic surgery and were expected to receive prophylactic anticoagulant therapy after the operation. We measured homocysteine concentrations before the operation, and during and after coumarin therapy. In a second study group, we measured homocysteine concentrations in 12 healthy volunteers who were treated with oral anticoagulants.

Results

Mean homocysteine concentrations increased 6% (95% CI 2% to 10%) during the treatment with coumarin derivatives. This corresponds with 1 µmol/l increase in homocysteine concentration. After the anticoagulant treatment period the concentrations decreased again. We calculated that this slight increase does not influence the interpretation of epidemiological studies. No influence on homocysteine concentrations was observed: decrease 3.6% (~0.6 µmol/l) (95% CI –17.5% to 8.5%) after 13 weeks of treatment with anticoagulants in healthy volunteers.

Conclusion

We conclude that there is no important effect of anticoagulation on homocysteine concentrations.

Introduction

Homocysteine is an aminoacid, formed after demethylation of methionine. It is either transsulphurated to cysteine or remethylated to methionine. Increased homocysteine concentrations are associated with arterial and venous thrombosis[1-4]. The association between homocysteine levels and arterial and venous thrombosis has been established in prospective studies, but mainly in retrospective case-control studies. In several of these studies on venous thrombosis, the patients were on treatment with anticoagulants at the time of venapuncture. In studies on arterial vascular disease many patients are also using coumarin derivatives, even in a prospective design. If anticoagulants influence the homocysteine concentrations, relative risk estimates will have been overestimated or underestimated. An influence of anticoagulants on homocysteine values could interfere with the outcome of these studies, especially since the odds ratios in the presented studies are usually small. To our knowledge there is no theoretical reason to believe that anticoagulants do influence homocysteine values. The aim of our study was to assess whether coumarin derivatives affect plasma homocysteine concentration *in vivo*. For this we measured plasma homocysteine concentrations before during and after anticoagulant treatment in two study groups. The first group underwent surgery and was treated with anticoagulants as prophylaxis; the second group were healthy volunteers who participated in a pharmacologic study.

Methods

Patient selection

We selected two study groups to assess the influence of coumarins on homocysteine. The first group were patients selected from the department of Orthopedics of the Leyenburg Hospital. Patients were all waiting for a hip- or knee-replacement operation and were asked to participate in this study. They were scheduled to take coumarin derivatives (acenocoumarol or phenprocoumon) until 3 months after the operation. Patients were excluded if they were using drugs that influence homocysteine- or folate concentrations (vitamin B, folic acid, methotrexate, phenytoin). 64 Consecutive patients were enrolled. Of these 64 patients, 24 patients were excluded for the following reasons: missing blood samples due to not showing up at follow-up visits, continued use of oral anticoagulant therapy, use of vitamin B after the start of the study, previously unmentioned use of oral anticoagulant therapy before the start of the study. Homocysteine concentrations of the remaining 40 patients (9 male, 31 female; age 47-88 years, median 71) were used for the analysis.

A second study was carried out in 12 healthy volunteers (6 men, 6 women, age 25-31 years) who took part in a study to evaluate the effect of low-dose vitamin K on INR levels. All volunteers were treated with coumarins (acenocoumarol) and adjusted to an INR of 2.0. The subjects were first adjusted to a stable INR (week 1-4) and subsequently supplemented with increasing doses of synthetic vitamin K_1 (in tablet form) over a 7-week period (weeks 5-11). Each K_1 dose was taken daily for a one-week period (Monday to Sunday), and in successive weeks the dosage was increased in increments of 50 μg K_1 over the range 50 μg to 300 μg, increasing to 500 μg K_1 for the final week. After the vitamin K supplementation period was a two week wash-out period (week 12 and 13).

Blood sampling and homocysteine measurements

Non-fasting blood (acidic citrate, Biopool, Stabilyte®) was taken three times from each patient in the first group of patients: before the operation, two months after the operation (on coumarin derivatives) and two months after cessation of the coumarin therapy. Blood was centrifuged within 30 minutes after collection at 2000*g* for 10 minutes. Plasma was separated and stored at -30ºC until serial determinations. In a subset of the patients blood was collected in EDTA (Vacutainer®, Beckton&Dickinson) for determination of folate and vitamin B 12 concentrations, according to standard techniques.

In the second group we determined homocysteine concentrations in standardized non-fasting morning samples (EDTA plasma). Blood was centrifuged within 30 minutes and stored at –20ºC. Samples were taken at the start of the study (before coumarin therapy was started), after week 4 (before the start of the low dose vitamin K), after week 10 (after combined vitamin K and coumarin treatment), and after week 13 (after the wash-out period of the vitamin K). The inclusion of this study group gives us the possibility to study both the effect of the drug medications as well as the anticoagulant effect on homocysteine levels. Details of the second study group have been published by Schurgers *et al.*[5].

Homocysteine concentrations of both study groups were determined using an automated high-performance liquid chromatography with reverse phase separation and fluorescence detection (Gilson 232-401 sample processor (Gilson Medical Electronics Inc., Middleton,WI), Spectra-Physics 8800 solvent delivery system and Spectra-Physics LC 304 fluorometer (San Jose,CA)), according to the method described by Fiskerstrand *et al.*[6] with some modifications[7].

Folate and vitamin B12 concentrations are determinants of the homocysteine concentration[8]. We measured folate and B12 with IMx Automated Immunoassay Analyzer (Abbott®) in a subset of patients of the surgery group.

Statistics

Paired-samples T-tests were used to compare geometric means of homocysteine, vitamin B12 and folate concentrations. Results are presented in percentages with 95% confidence intervals.

The study protocol was approved by the Leyenburg Ethics Committee for the surgery group. For the second study group, approval was granted by the Medical Ethics Committee of the University of Maastricht. All persons gave their informed consent according to the revised Helsinki declaration (1975).

Results

Figure 5.1 shows the individual values of the mean homocysteine concentration before during and after coumarin therapy in 40 patients. Geometric mean homocysteine concentration during coumarin therapy was 6% higher than before the start of coumarin therapy (95% confidence interval (CI) 2% to 10%; p=0.01). Absolute values increased from 16.4 µmol/l to 17.4 µmol/l. After cessation of the coumarins homocysteine decreased 4% (95% CI –1% to 10%; p=0.1) (absolute values 17.4 µmol/l to 16.7 µmol/l). The geometric mean difference between the concentrations before and after coumarin therapy was 1% (95% CI –4% to 7%; p=0.6).

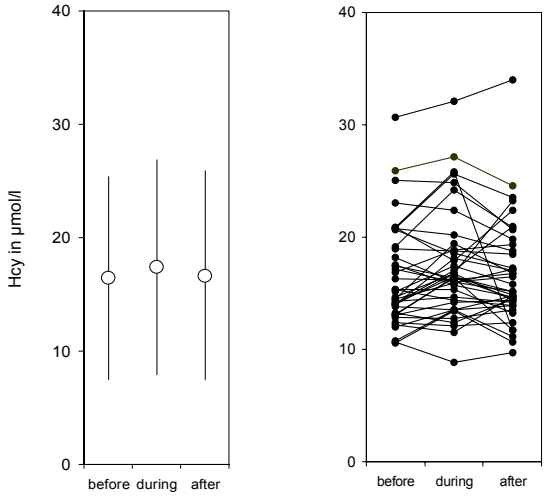

Figure 5.1 Mean homocysteine concentrations (Hcy) ± 2SD and individual homocysteine concentrations before the start of coumarin therapy, during coumarin therapy and after coumarin therapy in a group of surgery patients. See text for values.

Although the possible effect of anticoagulans on homocysteine values is very low, we analyzed if the 1 µmol/l increase in homocysteine concentration found in the surgery group was indeed due to treatment with anticoagulant drugs, we measured homocysteine concentrations in 12 volunteers who were treated with oral anticoagulants for 13 weeks. We found that geometric mean homocysteine concentrations decreased with 1.6% (95% CI –3.9% to 7.0%; p=0.5) after 4 weeks of treatment with oral anticoagulants, subsequently increased after vitamin K (2.9% (95% CI –5.2% to –0.4%); p=0.02), and decreased again after the wash-out of vitamin K (4.7% (95% CI –2% to 11%; p>0.05) The absolute values were 10.7, 10.1, 10.8, and 10.0 µmol/l, respectively. The individual concentrations and the mean INR levels in the study are shown in Figure 5.2. We conclude that there is no influence of anticoagulants on homocysteine values in healthy volunteers.

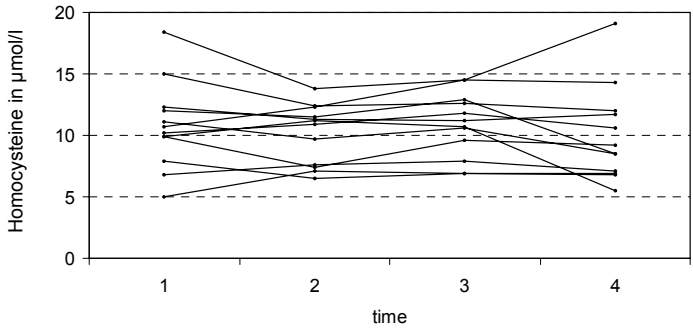

Figure 5.2 Homocysteine concentrations in 12 healthy volunteers treated with oral anticogulants and low-dose vitamin K.
1 = week 0. Before coumarins, mean INR=1.0
2 = week 4. After 4 weeks of coumarin therapy, mean INR=2.1
3 = week 10. After 11 weeks of coumarin therapy in combination with increasing doses of vitamin K, mean INR=1.4
4 = week 13. After the wash-out period of vitamin K, mean INR=1.9

We determined plasma vitamin B12 and folate concentrations in a subset of 27 surgery patients. No influence on vitamin B12 was observed (data not shown). However, folate level declined slightly (mean value from 6.7 nmol/l to 5.7 nmol/l), but not significantly (13%, 95% CI -3 to 27%; p=0.1). This was, however not associated with anticoagulant drugs since we saw no recovery after anticoagulant withdrawal but even a further decline from 5.7 nmol/l to 5.2 nmol/l (10 %, 95% CI –3% to 24%; p=0.1).

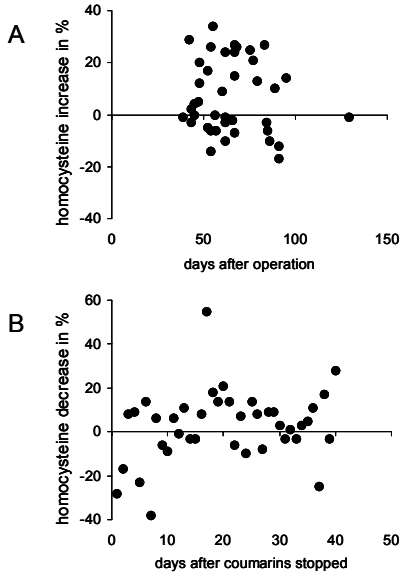

Figure 5.3 Percentile increase of homocysteine levels while on coumarin therapy in relation to the time interval passed since the operation(A) and percentile decrease of homocysteine levels after cessation of coumarin therapy(B)

Discussion

In this study we found a slight increase in homocysteine concentration in patients on prophylactic anticoagulant therapy for surgery. However, this effect was very small with a difference of 6% only, which corresponds to 1 µmol/l. In a group of healthy volunteers we found no influence of anticoagulants on homocysteine values at all.

The question is whether the slight increase in the surgery group was found due to coumarin use, or as an effect of the operation. We hypothesized that if there is an effect of the operation, it will diminish in the course of time. We compared the percentile increase in homocysteine concentration between the first (before coumarins) and the second (on coumarins) blood sample with the time elapsed after the operation. As shown in Figure 5.3A there was no relation between times elapsed after the operation and the percentile increase in homocysteine concentration (r=-0.1). In addition, we also determined the decrease in homocysteine concentration after the cessation of coumarins in relation to the time elapsed. Also, if the rise would be an effect of the coumarin use, the

percentile decrease after cessation of the coumarins would be greater when more time has passed. However, we found no relation between the time elapsed after the cessation of coumarins and the percentile decrease (Figure 5.3B) (r=0.07). A control group having similar surgery without anticoagulants is not possible for ethical reasons. A control group having a different form of thrombosis prophylaxis - like heparins – could have given information on the possible effect of different forms of anticoagulant treatment on homocysteine values versus the surgery procedure per se, but was not available.

The effect we observed in the surgery group was much smaller than the effect of coumarin use on homocysteine levels as found by Murua *et al.*[9]. They found a median difference of 9 µmol/l between a group of chronically anticoagulated patients with mitral valve replacement, atrial fibrillation and dilated cardiomyopathy in comparison to a group of patients with atherosclerosis who were not treated with coumarins. The authors concluded that this increase in homocysteine concentration was due to the lower folate levels in the patients on coumarins. The difference between the observation of Murua *et al.* and this study might be explained by the duration of coumarin treatment. Our study was aimed at analysing anticoagulants per se interfering with homocysteine values. Following patients for a longer period of time could coincide with a change in other variables such as folate levels observed by Murua.
The slight increase in homocysteine levels we found in the surgery group can be influenced by several factors. First, all our patients had a knee or hip replacement the day after the first blood sample was drawn. The use of nitrous oxide during operations can cause increments in homocysteine by cobalamin dependent blockage of methionine synthase[10-12], an enzyme necessary for the re-methylation of homocysteine. It has been shown to affect homocysteine concentrations up to one week postoperatively[12], but it is not known whether this effect lasts longer. When nitrous oxide is not used as an anesthetic, homocysteine is shown to remain stable[11] or even decrease[13]. Long-term effects of operations on homocysteine concentrations have not been reported. We reasoned that the effect would diminish in the course of time. The data shown in Figure 5.3A do not support the hypothesis that homocysteine concentrations increase due to a late effect of the operation: there is no relation between the time elapsed since the surgery and the increase in homocysteine, making an effect of aneshtesia on homocysteine concentrations unlikely.
Despite the fact that the influence on homocysteine concentration was very low in the surgery group, people who underwent this kind of major surgery, may have alterations in dietary habits, e.g. reduced folic acid and vitamin B12 intake. We did not observe an influence on vitamin B12 levels but a slight decline in plasma folate levels. The folate concentrations decreased even further after withdrawal of anticoagulants. Possibly, folate levels are an inpact

determinant of homocysteine values after surgery. This mechanism would fit to the analysis of Murua *et al.*[9].

Since we observed a minor effect in the surgery group, we subsequently studied the effect of coumarin treatment on homocysteine levels in a group of healthy volunteers. This way we were able to separate the effect of coumarins and surgery. In this group we found no effect in homocysteine concentrations during anticoagulant treatment. Since we found no effect of anticoagulant use in the healthy controls it is most plausible that the small effect in the surgery group was caused by clinical circumstances and not to the anticoagulant per se.

Many interactions of drugs and homocysteine concentrations have been described. Examples of these drugs are: B-vitamins, anti-folates like methotrexate, anti-epileptic drugs like phenytoin, the nitrous oxide and estrogens[8]. Most of these interactions are a result of direct influence of the drug on the metabolic pathways involved in homocysteine metabolism. As far as we know, the metabolic pathways of homocysteine and coumarins are not interchanged, and our results support the conclusion that there is no interaction.

Although an influence of coumarins on homocysteine levels is unlikely, we calculated wether a slight change in homocysteine levels as observed in the surgery group would affect the risk estimates in case-control studies in which only the cases used anticoagulant therapy. When homocysteine levels increase during anticoagulant therapy, more cases will have hyperhomocysteinemia and fewer cases will have a normal homocysteine concentration, resulting in a falsely elevated odds ratio. We calculated the impact of the 6% increase we found in our surgery group and we obtained correction factors of 0.89 for the 75th, 0.88 for the 90th and 0.88 for the 95th percentile (using reference values of a case control study we performed earlier[2]). Thus, if an effect of coumarins on homocysteine concentration would indeed exist - at a level 1 µmol/l increase - this would have had only a very small effect on the odds ratios from comparing studies in which coumarins were used in patients and not controls.

Usually, the increase in homocysteine concentration in patients at risk for vascular disease is expressed as odds ratio per 5 µmol/l. For venous thrombosis this odds ratio is approximately 1.6[1], for stroke and ischemic heart disease 1.4 and 1.2 respectively[3]. A change of 1 µmol/l in homocysteine concentration, as we found in the surgery group, corresponds with a risk ratio of approximately 1.01. Therefore, when homocysteine concentrations are measured in a screening program for cardiovascular risk factors in individual patients, the use of anticoagulant therapy during this screening has no significant impact on the risk estimate. Moreover, because results from adequate intervention trials in atherosclerosis are conflicting[14-16], and the

results of intervention trials in venous thrombosis are still limited[17] the benefit of determination of individual homocysteine concentrations remains unclear.
In conclusion, we found a slight but not relevant increase in homocysteine values in patients who were on anticoagulant therapy because of orthopedic surgery, and no influence in healthy volunteers taking anticoagulants for scientific reasons. We conclude that anticoagulants per se do not influence homocysteine values at a level to consider relevant in epidemiological studies nor in individual patient care.

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Chapter 6

The elevated risk for venous thrombosis in persons with hyperhomocysteinemia is not reflected by the endogenous thrombin potential

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Introduction

Several case control studies and a recent prospective study showed that in patients with (idiopathic) venous thrombosis mild hyperhomocysteinemia (HH) can be observed 2-3 times more frequently than in controls[1-3]. The pathogenetic explanation for this clinical observation is not known. In principal a thrombotic tendency can originate in the blood, in the vessel wall or at the level of thrombocytes. The question that we wanted to answer was whether the thrombotic tendency that might accompany HH is caused by a higher capacity of these persons to generate thrombin. The plasmatic component of a thrombotic tendency might be reflected in the capacity of the platelet poor plasma to generate thrombin. This capacity can be assessed by measuring the endogenous thrombin potential (ETP), i.e. the surface under the thrombin generation curve[4-6]. It has been shown that the ETP is significantly increased in such plasma based thrombotic tendencies as deficiencies in AT and mutated Factor V Leiden[6,7]. The influence of exogenous activated protein C (APC)[8,9] and exogenous thrombomodulin (TM)[10,11] on the inhibition of the ETP was recently shown and was used to screen for a deficient protein C pathway. In order to see whether a plasmatic component can explain the thrombotic tendency in HH, we tested the possible relationship between the concentration of homocysteine (tHcy) and thrombin generation in a group of healthy controls, without any venous thrombotic events in the past.

Methods

Blood samples of 30 persons with elevated levels of tHcy (>18 µmol/l; mean: 22.7 mmol/l) were selected. Samples of 30 persons matched for age and sex with normal tHcy levels (mean: 14.2 µmol/l) were used as controls (for details on study group selection see ref. 12). Homocysteine values of >18 µmol/l are clearly associated with increased risk for venous thrombosis[13,14]. We deliberately did not include patients with venous thrombosis in the past, so as to exclude the possibility of plasma changes caused by the thrombotic process itself. We determined the ETP under standard conditions (extrinsically and intrinsically) and in the presence of exogenous APC or TM. The assay method for determining the ETP is based on the continuous monitoring of thrombin formation using a slow reacting thrombin substrate essentially as described earlier[4] which method has been adapted for high throughput screening on a Cobas centrifugal analyzer[6]. The anticoagulant response towards TM was expressed as the thrombomodulin ratio (TMR); TMR: (ETP+TM/ETP-TM)pool/ (ETP+TM /ETP-TM)sample. The anticoagulant response towards APC was

expressed as the APC sensitivity ratio (APC-sr): (a2M–IIa+APC/a2M–IIa-APC)sample / (a2M–IIa+APC/a2M–IIa-APC)pool.

Results

The data on the subjects under study are given in Table 6.1. There was a narrow association between the intrinsic and extrinsic ETP. The Pearson correlation is 0.876 (p=0.001) (data not shown). However, for both intrinsic ETP and extrinsic ETP we found no correlation with tHcy. Comparing the two different groups no difference was present between those with normal tHcy levels and those with elevated tHcy levels. The intrinsic ETP in those with elevated tHcy was 412 (99% of reference plasma) similar to those with normal tHcy. The extrinsic ETP was 100% of reference plasma in those with eleveated tHcy and 106% of reference plasma in those with normal tHcy. There was clearly no association between tHcy and TMR (coefficient is 0.04; p=0.74) and no difference between the group with high tHcy and those with normal tHcy was observed. Also no difference in APC-sr ratio could be observed between those with high and normal tHcy levels.

Table 6.1 Homocysteine and ETP values of persons under study.

	normal tHcy	high tHcy	p-value
tHcy mean (µmol/l)	14.2	22.7	
tHcy range	8.4 – 16.5	18.0 – 49.8	
Mean age (range)	55.2 (23 - 82)	55.1 (23 - 80)	
Male (N)	15	15	
Female (N)	15	15	
ETP intrinsic	412 +/- 72 nM.Min	412 +/- 66 nM.Min	0.6
ETP extrinsic	419 +/- 65 nM.Min	395 +/- 56 nM.Min	0.15
APC-sr	1.37 +/- 0.33	1.41 +/- 0.51	0.75
TMR	0.83 +/- 0.15	0.86 +/- 0.18	0.37

Discussion

Since no association of the ETP and homocysteine levels was observed and no influence of homocysteine on the ETP in the presence of APC or TM, our data do not support the idea that HH acts via the plasmatic coagulation system.

Others suggested a role for factor V or activated protein C. In addition an enhanced turnover or diminished formation of thrombomodulin has been suggested though not supported by all studies (reviewed in 1 and 15). It should be realized however that most of the observations were made in *in vitro* systems and that in the *in vitro* experiments very high levels of (free) homocysteine – up to 10 mmol/l – were used. These values differ far from the *in vivo* situation and it is questionable in our opinion whether these *in vitro* experiments represent the clinical situation. Not finding an association between mild HH and the ETP renders a direct influence of HH on plasmatic thrombin generation improbable. Therefore other factors might be relevant such as the fibrinolytic pathway, enhanced tissue factor activity, enhanced platelet aggregation, increased platelet adhesion on endothelial cells, abnormal nitrogen oxides, abnormal endothelium-derived relaxing factor and inhibition of von Willebrand factor production[1,15]. Most of the studies supporting these hypotheses are however again limited by the high levels of homocysteine used in the *in vitro* experiments. It has also been shown that homocysteine might induce altered gene expression in endothelial cells, genes that might possibly be related to the process of thrombosis[16,17]. Until now –to our knowledge– there is however no clear parameter observed in man that might be a clue for the pathogenetic process involved in the association of mild HH and venous thrombosis. We feel such a parameter is urgently needed to proof that the epidemiological association between HH and venous thrombosis can more likely be interpreteted as a causative one. Furthermore such a parameter would be very helpful in treatment strategies for HH. Vitamins (folic acid) can easily correct HH but any antithrombotic effect of any treatment cannot be claimed yet[12,15,18].

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Chapter 7

Hyperhomocysteinemia as a risk factor for venous thrombosis in elderly patients

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Submitted for publication

Abstract

High plasma levels of homocysteine are a risk factor for venous thrombosis. Although thrombosis has a high incidence in elderly people, little is known about the risk of venous thrombosis related to hyperhomocysteinemia in the elderly .

We performed a case-control study with 426 patients with a first, idiopathic and objectively diagnosed deep-vein thrombosis or pulmonary embolism and 294 control subjects from the general population. All subjects were >65 years of age.

Mean homocysteine levels were higher in the cases (14.4 (95% CI 13.9 to 14.9) µmol/l) than in the controls (13.2 (95% CI 12.7 to 13.7) µmol/l). There was a linear relationship between quartiles of homocysteine concentration and thrombosis risk, with an odds ratio for the highest versus the lowest quartile of 1.7 (95% CI 1.1 to 2.7).

We conclude that mild hyperhomocysteinemia is a risk factor for venous thrombosis in the elderly.

Introduction

Venous thrombosis is a common disease, especially in the elderly. The incidence rises from approximately 25 / 100,000 / year at the age of 25 to 500 / 100,000 / year over the age of 80[1]. Immobility, malignancy and major surgery are well known environmental risk factors for venous thrombosis. These may in part explain why elderly patients are more at risk for developing venous thrombosis since the prevalence of these risk factors is higher at higher age, but it cannot explain the steep rise in incidence that is seen in the elderly.
High levels of homocysteine are an established risk factor for venous thrombosis. The association has been established in numerous case-control studies and three prospective studies[2]. Most of these studies did not focus on elderly patients. Since plasma homocysteine levels increase exponentially with age[3], homocysteine might play an important role in the development of venous thrombosis in this age group. Two previous studies have reported on the risk of hyperhomocysteinemia and the development of venous thrombosis and the relation with age. Den Heijer *et al*. found a sharp increase in the risk of thrombosis with age associated with hyperhomocysteinemia for both men and women[4]. The results of this study could not be confirmed by Tsai *et al*. who found no association between hyperhomocysteinemia and an increased risk of thrombosis at high age[5]. More studies on this subject are lacking. We therefore performed a case-control study among elderly patients to evaluate whether elevated homocysteine levels are a risk factor for venous thrombosis in this age group.

Patients and methods

Cases

Cases were selected from the screening of the VITRO (vitamin and thrombosis) study. The design of the study was described previously[6]. In short, the VITRO study is a randomized, placebo-controlled, double-blind trial with multivitamin B as secondary prevention of venous thrombosis in patients with hyperhomocysteinemia. Patients for the trial were selected from seven anticoagulation clinics in the Netherlands from February 1995 to June 2000. Anticoagulation clinics monitor the treatment of virtually all patients on coumarins in a well described geographic area. All patients who were registered for treatment for a first deep-vein thrombosis or pulmonary embolism

were eligible for screening and asked to donate an extra blood tube for homocysteine measurement.
In the current analysis we included patients from the anticoagulation clinics of The Hague and Rotterdam who were aged 65 years or older at the time of a first event of idiopathic venous thrombosis, which was diagnosed by objective methods (proximal deep-vein thrombosis by compression-ultrasonography or phlebography, pulmonary embolism by high-probability VQ-scanning, pulmonary angiography or spiral CT scanning). We considered as idiopathic events that were not preceeded by immobilisation (bed-rest, paresis, cask), major trauma or major surgery, vasculitis or intravenous catheters, or malignancy). Information on diagnostic methods and these risk factors were obtained from the treating physician.

Control subjects

Control subjects were selected from two general practices in The Hague and Rotterdam. In the Hague all persons of 65 years and older registered at this practice were asked by mail to participate. In the Rotterdam practice we randomly invited 500 persons of 65 years and older from all patients registered at the practice. They were eligible for participation in the absence of a previous venous thrombosis and malignancy. People with a malignancy were excluded from the analysis.

Blood handling

Blood was drawn before 10 a.m. in acidic citrate tubes (Biopool Stabilyte^{TM}). In a previous study we showed that acidic citrate stabilizes whole blood for measurement of homocysteine at 21°C[7,8]. Blood was stored at room temperature and was processed within 5 hours after collection. It was centrifuged for 10' at 2000*g* in a non-cooled centrifuge. The plasma was separated and stored at -30°C until determination of the homocysteine concentration.
Homocysteine was determined by automated high-performance liquid chromatography (HPLC) with reverse phase separation and fluorescent detection (Gilson 232-401 sample processor (Gilson Medical Electronics Inc., Middleton,WI), Spectra-Physics 8800 solvent delivery system and Spectra-Physics LC 304 fluorometer (San Jose,CA)), according to the method described by Fiskerstrand *et al.*[9] with some modifications[10,11]. Homocysteine concentrations were multiplied by 10/9 as correction for the fluid present in the blood tube prior to collection.

Data analysis

We calculated age and sex specific quartiles of homocysteine. The odds ratios for the second to the fourth quartile (with the first quartile as a reference category) was calculated with a logistic regression model.

Results

At the anticoagulation clinics of The Hague and Rotterdam we screened 2821 patients for the VITRO study. Of these, 1195 were above 65 years. We obtained information about circumstances and diagnostic methods of the venous thrombosis of 1097 patients. Of these, 110 patients had a recurrence, and 572 patients had a first, idiopathic venous thrombosis of which 146 were not objectively diagnosed according to our entry criteria. This left 426 patients who met the participation criteria for the current study.
From 1045 persons invited by mail to participate as control subject, 294 consented to participate. Baseline characteristics of the patients and controls are shown in Table 7.1. There were more men in the patient group than in the control group (46% vs. 41%) and the mean age was higher in the patients group (74.5 vs 73.2 years).

Table 7.1 Baseline characteristics of the patients and controls.

	patients	controls
number	426	294
men / women	196 / 230	120 / 174
median age (range) in years	74 (65-93)	72 (65-96)
mean homocysteine (range) in µmol/l	13.2 (12.7-13.7)	14.4 (13.9 – 14.9)
thrombosis		
deep-vein thrombisis	255	
pulmonary embolism	135	
both	36	

The distribution of the homocysteine concentrations of patients and control subjects is shown in Figure 7.1. Homocysteine was higher in men (1.5 (95% CI 0.7 to 2.4) µmol/l) and increase with age both in men (3.0 (95% CI 0.8 to 5.2) per decade) and women (2.2 (95% CI 1.3 to 3.2) per decade). Therefore, we calculated the odds ratios for thrombosis for age- and sex- specific quartiles with the lowest quartile as the reference quartile (Figure 7.2). We found a linear dose-response relationship with an odds ratio for the highest versus the lowest

quartile of 1.7 (95% CI 1.1 to 2.7). There was no apparent difference in risk in the different age and sex groups. Stratification for type of thrombotic event did not show clear differences in risk for deep-vein thrombosis (odds ratio top versus bottom 1.9 (1.2 to 3.0)), pulmonary embolism (1.5 (0.8 to 2.7) or both (1.4 (95% CI 0.5 to 3.8).

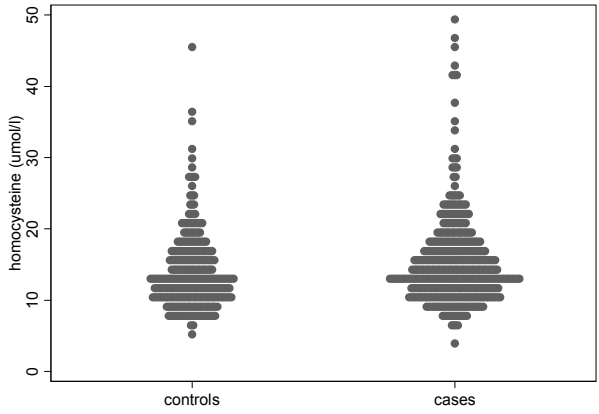

Figure 7.1 Distribution of homocysteine concentrations of controls and cases (in µmol/l).

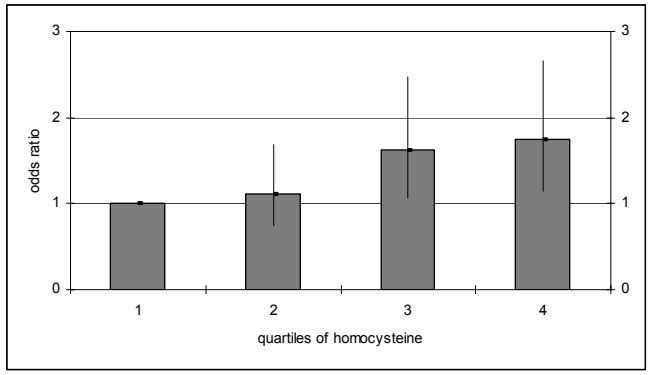

Figure 7.2 Thrombosis risk for age- and sex specific quartiles of homocysteine concentrations.

Discussion

We found that the homocysteine concentration in plasma is a risk factor for venous thrombosis in elderly individuals as it is among younger people.

Furthermore, we found a graded increase in the risk with increasing homocysteine concentrations.

The risk estimate that we report in this study lies between that of our earlier study[4] and the study of Tsai and colleagues[5]. In a case control study with 269 patients with a deep-vein thrombosis and 269 age- and sex- matched controls we reported an increase in relative risk for hyperhomocysteinemia from 0.7 (95% CI 0.1 to 4.0) under the age of 30, up to 5.5 (95% CI 1.2 to 5.2) over the age of 50. This graded increase in risk was seen in both men and women.

However, Tsai and colleagues looked at homocysteine as risk factor for venous thromboembolism in the LITE study, a nested case-control study[5]. They found an overall odds ratio of 1.55 (95% CI 0.93 to 2.58) for the highest versus the lowest quintile. They report an attenuation of the association with increasing age, and absence of any excess risk above 65 years.

In the current study the risk estimate, as an odds ratio, is very similar to those reported in studies in younger patient groups. Even though the odds ratios we calculated are similar to those in studies of younger subjects, this may imply that the absolute effect of hyperhomocysteinemia is greater among the elderly, because the incidence of thrombosis is much higher[1].

In conclusion, homocysteine is a risk factor for venous thrombosis in patients above 65 years old.

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Chapter 8

Homocysteine lowering by B vitamins and the secondary prevention of deep-vein thrombosis and pulmonary embolism. A randomized, placebo-controlled, double blind trial

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Abstract

The VITRO (VItamins and ThROmbosis) study investigated the effect of homocysteine lowering by daily supplementation of B-vitamins on the risk reduction of deep-vein thrombosis and pulmonary embolism. Patients between 20 to 80 years old with a first objectively confirmed proximal deep-vein thrombosis or pulmonary embolism in the absence of major risk factors and a homocysteine concentration above the 75th percentile of a reference group were asked to participate (hyperhomocysteinemic group). A similar study was conducted in a random sample of patients with a homocysteine below the 75th percentile of the reference group (normohomocysteinemic group). After informed consent patients were randomized to daily multivitamin supplementation (5 mg folic acid, 50 mg pyridoxine and 0.4 mg cyanocobalamin) or placebo and were followed for 2.5 years. End-points were objectively diagnosed recurrent deep-vein thrombosis or pulmonary embolism.

A total of 701 patients were enrolled (360 in the hyper- and 341 in the normohomocysteinemic group). The number of recurrent events of venous thrombosis was 43 out of 353 in the vitamin group (54/1000yr) and 50 out of 348 in the placebo group (64/1000yr). The hazard ratio associated with vitamin treatment was 0.84 (95% CI 0.56 to 1.26): 1.14 (95% CI 0.65 to 1.98) in the hyperhomocysteinemic group and 0.58 (95% CI 0.31 to 1.07) in the normohomocysteinemic group. The results of our study do not show that homocysteine lowering by B-vitamin supplementation prevents recurrent venous thrombosis.

Introduction

Plasma homocysteine levels are associated with an increased risk of deep-vein thrombosis and pulmonary embolism. Up to now 24 case-control studies have been published with an overall relative risk for venous thrombosis of 1.60 (95% CI 1.10 to 2.34) for a 5 µmol/l higher homocysteine level[1]. Moreover, three prospective studies showed an overall relative risk for venous thrombosis of 1.27 (95% CI 1.01 to 1.59) for a difference of 5 umol/l[1]. Recent meta-analyses on the effect of the MTHFR 677TT genotype on cardiovascular disease (2) and venous thrombosis[1,3] showed a modest increase in risk, supporting a hypothesis that homocysteine levels are causally related to thrombotic risk.
Elevated homocysteine levels can be easily treated with B-vitamin supplementation (folic acid, vitamin B6 and vitamin B12). Daily use of folic acid gives a 25% reduction in homocysteine levels even at low doses of 0.5 mg[4,5]. The question is whether lowering of homocysteine by use of B-vitamin supplementation also lowers the risk for venous thrombosis.
In the VITRO (VItamins and ThROmbosis) study, the primary aim was to investigate the effect of a combination preparation of 5 mg folic acid, 50 mg of pyridoxine and 0.4 mg cyanocobalamin in the secondary prevention of deep-vein thrombosis and pulmonary embolism in patients with a first event of venous thrombosis and hyperhomocysteinemia in a randomized, double-blind and placebo controlled setting.
A secondary aim was to study the effect of vitamin supplementation in patients with a first event of venous thrombosis and a 'normal' homocysteine concentration in an identical setting[6].

Patients and methods

Study participants

Patients were selected through anticoagulation clinics in The Netherlands. Anticoagulation clinics monitor the anticoagulant treatment of virtually all patients in well-defined geographical areas. Participating anticoagulation clinics asked all patients with a first venous thrombosis to donate a blood sample for homocysteine determination. Patients with a homocysteine plasma concentration in the top quartile of its distribution in the general population (homocysteine ≥12.6 µmol/l)[7] and who met the entry criteria formed the hyperhomocysteinemic group. Enrollment started in March 1996. Because the rate of inclusion was lower than expected, the trial was extended in 1998 with the Thrombosis Centers of Milan and Vienna who included patients with homocysteine above the 75th percentile based on reference population of the respective countries (homocysteine ≥10.6 µmol/l in Milano and homocysteine

≥8.5 µmol/l in women and ≥10.4 µmol/l in men in Vienna). The latest patient was included in May 2001. Parallel to the study in the hyperhomocysteinemic group we performed a study in the normohomocysteinemic group, which was done only in the Netherlands. During the study there was no folate fortification in these three countries.

For all patients who consented in donating blood for homocysteine measurement information was retrieved from the general practitioner or specialist of the patients about the diagnosis and circumstances in which patients developed their thrombosis. Patients were eligible when they had objectively confirmed proximal deep-vein thrombosis or pulmonary embolism in absence of major risk factors (major surgery, known malignant disease, pregnancy and puerperium or immobility for more than three weeks), are aged between 20 to 80 years at time of diagnosis and without obligatory use of vitamin B. When patients met all entry criteria, they were asked to give their informed consent in accordance with the current revision of the declaration of Helsinki (2000).

Randomization and intervention

Eligible patients were randomized to receive high-dose multivitamin daily or identicalappearing placebo. The high-dose multivitamin capsule contained 5 mg folic acid, 0.4 mg cyanocobalamin and 50 mg pyridoxine. The randomization was performed with 4 and 6 random permuted blocks, stratified by homocysteine status (hyperversus normohomocysteinemia), sex and by anticoagulation clinic or study center. The study medication was based on an earlier study on the homocysteine lowering effects of B-vitamins[5]. The medication was tested for stability for the duration of the trial through determination of the vitamin contents of the vitamin capsules. The ranges found during 42 months were 0.4-0.5 mg/capsule for cobalamin, 48.3-59.1 mg/capsule for pyridoxine and 5.1-7.0 mg/capsule for folic acid. Placebo's were made for this trial and capsules were identical for both placebo and vitamins.

Duration of treatment and follow-up was intended for 2.5 years. Participants were seen (after overnight fasting) at the start of the study (before randomization) and 3, 6, and 24 months after randomization. Blood was collected at each visit, for determination of homocysteine. Patients received their study medication at these follow up visits or by mail every 3 or 6 months. The participants started with their study medication as soon as they were randomized i.e. within the period of anticoagulant treatment in order to achieve the homocysteine lowering effect in the vitamin group before the cessation of anticoagulant treatment. Compliance of the drugs was monitored by measuring homocysteine levels.

End-points

The primary endpoint of the study was recurrent symptomatic DVT or recurrent PE. This endpoint was defined as the decision of the treating physician to restart anticoagulant medication. The treating physician was not informed about study medication or homocysteine concentration. Because it might be difficult to make an accurate diagnosis of recurrent deep-vein thrombosis or pulmonary embolism because of residual thrombi, we provided a tool for the treating physicians to make the diagnosis of recurrent deep-vein thrombosis more accurate. In patients with a deep-vein thrombosis of the leg a compression ultrasonography (CUS) was done 3 months after the thrombotic event. If a residue of the old thrombus was seen on the CUS, the CUS was repeated 6 and if necessary 12 months after the thrombosis. In patients with a PE, CUS of both legs was performed to exclude a DVT. The ultrasonographies were performed in one hospital or institution in every participating center. The results of these tests were noted down in a so-called 'patient passport', a booklet which patients were instructed to take with them if they visited their physician with symptoms of a recurrent thrombosis. By using this passport, data on residual thrombosis were available, even if the patient visited another hospital with complaints of recurrent thrombosis. The recommended definition of 'recurrent DVT' was when a previously normal or normalized venous segment could not be compressed with CUS, or when there was an increment in the diameter of residual thrombus with 4 mm[8,9]. The diagnosis of recurrent PE was according to standard clinical practice.

Laboratory measurements

To screen patients with first-time venous thrombosis for hyperhomocysteinemia, homocysteine has to be measured in a large number of patients. To avoid homocysteine increase after blood sampling, we used blood collection tubes with acidic citrate as anticoagulant[10]. After entering the intervention study blood was taken at 0, 3, 6 and 24 months after start of the study. This blood was collected after an overnight fast in EDTA-tubes and directly placed on ice and centrifuged within one hour. The total homocysteine concentration in EDTA-plasma was measured in one central laboratory (Laboratory of Pediatrics and Neurology in Nijmegen) by an automated high-performance liquid chromatography method with reverse phase and fluorescent detection (Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorometer), essentially according to the method by Fiskerstrand *et al.*[11], with modifications[12].

Study size

Sample size was calculated for the hyperhomocysteinemic group: With alpha=0.05 and beta=0.2 and with an expected recurrence rate of 20% in patients with idiopathic thrombosis (based on the study of Eichinger *et al.*[13]) and hyperhomocysteinemia in 2.5 year, and a 50% risk reduction due to the vitamin therapy (based on a relative risk of more than two for hyperhomocysteinemia[7,13] and the assumption that a 90% of those with homocysteine levels above the 90th percentile could be reduced to less than the 90th percentile with multivitamin treatment[5], 155 patients in each treatment group were required in the hyperhomocysteinemic group[6]. It was decided to randomize the same number of patients in the normohomocysteinemic group. So the intended total sample size was 620.

Statistics

We compared the high-dose vitamin group and the placebo group with respect to age, sex, type of first event (DVT versus pulmonary embolism), initial homocysteine levels for both the hyperhomocysteinemic patients and normohomocysteinemic patients respectively.

Relative risk estimates (hazard ratios) and their 95% confidence intervals (CI) were calculated with a Cox proportional hazard model to assess the effects of high-dose multivitamin supplementation. Variables included in the model were treatment regimen (vitamin versus placebo) and the variables on which the randomization was stratified i.e. sex, anticoagulation clinic and initial homocysteine levels (hyperhomocysteinemic or normohomocysteinemic).

The primary analysis was an intention-to-treat analysis starting at the day of randomization and a follow-up of 2.5 year. We did an on-treatment analysis with restriction of the observation time to the time that patients had reported to take their capsules. A second on-treatment analysis was performed by stratifying the homocysteine reduction in three categories (more than 50% reduction, 50-0% reduction and no reduction in homocysteine level) and calculating the hazard ratio for the first two categories compared to no homocysteine reduction.

Because the treatment regimen started while patients were on anticoagulation treatment (which has a great influence on the risk of recurrence) we also did an analysis without taking in to account the recurrences that occurred before two months after cessation of anticoagulation . In all these three models the data of randomization (and start of the treatment regimen) was the starting time in the Cox model. Finally we used a Cox model with the date of cessation of anticoagulation as starting time.

To assess the role of baseline homocysteine levels as a risk predictor of recurrent events we also performed a Cox model with homocysteine as

continuous variable (in µmol/l) and age, sex and study medication as covariates.

Results

The participating anticoagulation clinics screened 4382 patients (Figure 8.1). Of these patients, 2000 had a homocysteine plasma concentration ≥12.6 µmol/l (75th percentile in a general Dutch population). 1522 of these 2000 patients did not meet the entry criteria and 153 refused participation. The remaining hyperhomocysteinemic patients (n=325) were randomized in the hyperhomocysteinemic group. The Thrombosis Centers of Milan and Vienna included an additional 35 patients with homocysteine above the 75th percentile based on reference population of the centers. Of the 4382 patients screened in the Netherlands 2382 had homocysteine values below 12.6 µmol/l. 1886 patients did not meet the entry criteria or were randomly excluded and 155 refused participation. A total of 341 patients were randomized in the normohomocysteinemic group.

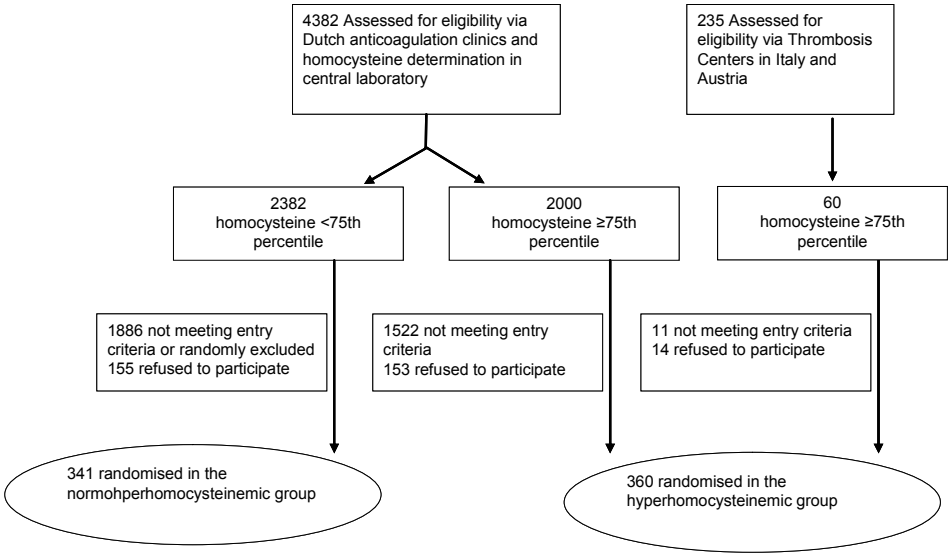

Figure 8.1 Study design

The baseline characteristics for the vitamin and placebo groups according to their homocysteine level (hyperhomocysteinemic and normohomocysteinemic) are shown in Table 8.1. The differences in homocysteine levels between the hyperhomocysteinemic and the normohomocysteinemic group based on the

homocysteine measurement at time of screening remained high at the start of the treatment study. The hyperhomocysteinemic group was slightly older than the normohomocysteinemic group and included more men, due to the use of a uniform cut-off value. However, the vitamin and placebo groups were very similar in both the hyper- and normohomocysteinemic groups.

Table 8.1 Baseline characteristics.

	Hyperhomocysteinemic group (n=360)		Normohomocysteinemic group (n=341)	
Variable	multivitamin (n=177)	placebo (n=183)	multivitamin (n=176)	placebo (n=165)
Sex (M/F)	103/74 (58/41%)	105/78 (57/43%)	80/96 (45/55%)	74/91 (45/55%)
Median age in years (range)	56.4 (18.1-79.9)	57.2 (17.9-79.8)	48.2 (20.2-75.5)	46.3 (19.1-78.5)
Type first event				
deep-vein thrombosis	119 (76%)	126 (69%)	97 (55%)	100 (61%)
pulmonary embolism	43 (24%)	40 (22%)	60 (34%)	51 (31%)
both	15 (8%)	17 (9%)	19 (11%)	14 (8%)
Median duration of anticoagulation in months (range) after randomization	1.6 (0-30)	1.8 (0-30)	1.5 (0-18)	1.6 (0-30)
Geometric mean baseline homocysteine in µmol/l 95% CI) [range]	15.1 (14.3-16.0) [6.3-84.8]	15.9 (14.9-17.0) [7.4-108.3]	9.0 (8.7-9.3) [4.0-23.0]	9.0 (8.7-9.3) [4.1-15.5]
Geometric mean homocysteine after three months in µmol/l 95% CI) [range]	8.5 (8.1-8.9) [4.1-21.3]	15.6 (14.5-16.8) [6.0-91.7]	6.5 (6.2-6.7) [2.9-11.6]	9.7 (9.4-10.1) [5.5-25.6]

We analyzed the effect of the vitamin/placebo treatment 3 month after start of the intervention. These data demonstrated no effect of placebo on the homocysteine values, whereas a 46% reduction of homocysteine values could be demonstrated in the hyperhomocysteinemic group and a 33% reduction was observed in the normohomocysteinemic group.

During the course of the study, 43 out of 353 (12.2%) patients suffered from a recurrent event of venous thrombosis in the multivitamin group and 50 out of 348 (14.3%) patients had a recurrent venous thrombosis in the placebo group.

Figure 8.2 shows the recurrent thrombosis cumulative incidence curves of patients treated with multivitamins versus those treated with placebo. The overall hazard ratio was 0.84 (95% CI 0.56 to 1.26), e.g. a risk reduction of 16% (95% CI –26 to 44). The hazard ratio associated with vitamin supplementation was 1.14 (95% CI 0.65 to 1.98) in the hyperhomocysteinemic group and 0.58 (95% CI 0.31 to 1.07) in the normohomocysteinemic group. The hazard ratio for men versus women was 1.6 (95% CI 1.05 to 2.45). There was no significant effect for the other covariates.

The results of the on-treatment analysis were similar to the intention to treat analysis (Table 8.2). However, when we stratified the homocysteine reduction

in three categories (more than 50% reduction, 50-0% reduction and no reduction in homocysteine level) we found a hazard ratio of 0.82 (95% CI 0.51 to 1.32) for a 50-0% reduction and 0.43 (95% CI 0.15 to 1.24) for a more than 50% reduction in homocysteine compared to no reduction.

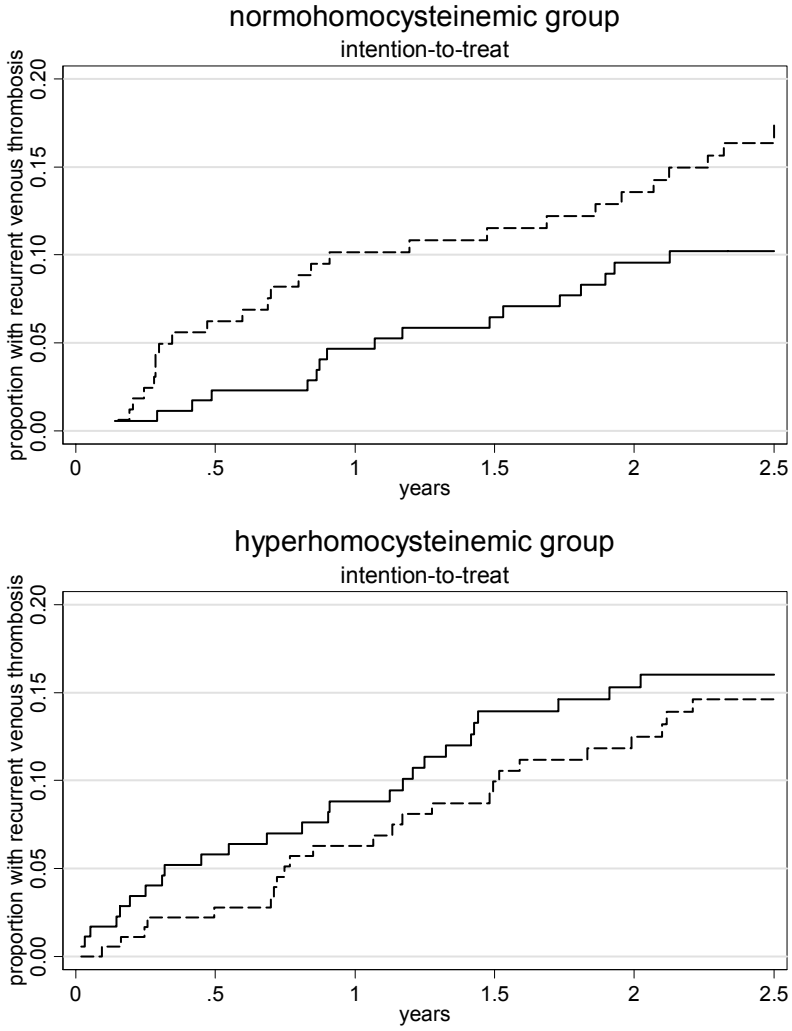

Figure 8.2 Recurrent thrombosis cumulative incidence in patients treated with multivitamin (——) or placebo (- - - -) in a hyperhomocysteinemic and a normohomocysteinemic group.

Because the treatment regimen started while patients were on anticoagulation treatment (which has a great influence on the risk of recurrence) we also did an analysis after exclusion of early recurrences (during anticoagulant treatment or within the first two months after cessation of anticoagulant treatment) (Table 8.2). This subgroup analysis gave similar risk estimates for the hyper- and normohomocysteinemic group. This was also seen in the fourth analysis in which we took the date of cessation of anticoagulation as starting time. in the Cox model.

Table 8.2 Incidences and relative risks for recurrent venous thrombosis

	Vitamin[a] n/py (ir%)	Placebo[a] n/py (ir%)	HR vitamin versus placebo[b]
Intention to treat analysis			
Hyperhomocysteinemic group	26/387 (6.7%)	24/403 (6.0%)	1.14 (0.65 to 1.98)
Normohomocysteinemicgroup	17/412 (4.1%)	26/373 (7.0%)	0.58 (0.32 to 1.08)
Overall	43/799 (5.4)%	50/776 (6.4%)	0.84 (0.56 to 1.26)
On treatment analysis			
Hyperhomocysteinemic group	24/338 (7.1%)	22/344 (6.4%)	1.13 (0.63 to 2.02)
Normohomocysteinemicgroup	16/363 (4.4%)	22/337 (6.5%)	0.65 (0.34 to 1.24)
Overall	40/702 (5.7%)	44/682 (6.4%)	0.88 (0.57 to 1.36)
Intention to treat analysis with exclusion of early recurrences[c]			
Hyperhomocysteinemic group	17/387 (4.4%)	21/403 (5.2%)	0.84 (0.44 to 1.60)
Normohomocysteinemicgroup	15/412 (3.6%)	20/373 (5.4%)	0.66 (0.34 to 1.30)
Overall	32/799 (4.0%)	41/775 (5.3%)	0.76 (0.48 to 1.21)
Intention to treat analysis beginning after cessation of anticoagulation			
Hyperhomocysteinemic group	23/338 (6.8%)	24/347 (6.9%)	0.98 (0.55 to 1.74)
Normohomocysteinemicgroup	17/379 (4.5%)	24/338 (7.1%)	0.62 (0.33 to 1.15)
Overall	40/717 (5.6%)	48/685 (7.0%)	0.80 (0.52 to 1.21)

[a] number of recurrences,py=person years, ir=annual incidence in %; [b] HR=hazard ratio (95% CI), adjusted for study center, sex and hyper-/normohomocysteinemia; c early recurrences: recurrences before two months after cessation of anticoagulation.

Although the duration of anticoagulant treatment was similar for the various groups, there was a relatively high number of early recurrences in the hyperhomocysteinemic vitamin group (9 events) compared with the hyperhomocysteinemic placebo group (3 events). In contrast, in the normohomocysteinemic group early recurrences occurred more often in the placebogroup (6 events) than in the vitamin group (2 events).

To assess the role of baseline homocysteine levels as a risk predictor of recurrent events we performed a Cox model with homocysteine as continuous variable (in µmol/l) and age, sex and study medication as covariates.The hazard ratio for recurrence associated with a 5 µmol/l higher homocysteine level at baseline was 1.13 (95% CI 1.05 to 1.20). This effect was similar in the

placebo group as in the vitamin group. The hazard ratio for a homocysteine concentration above the 90th percentile (20.1 µmol/l) was 1.8 (95% CI 1.1 to 3.2). Homocysteine levels were not associated with early recurrences.

Discussion

Our study is the first clinical trial on the effect of B-vitamins in the prevention of recurrent venous thrombosis. Our study shows that B-vitamin supplementation lowers homocysteine values but it doesn't show a risk reduction in recurrent venous thrombosis. Homocysteine at baseline is a modest risk factor for recurrent events.

The results of our trial showed a difference in effect in the hyperhomocysteinemic group compared to the normohomocysteinemic group. This difference in effect was contrary to what was expected and could not be biologically explained. Therefore we looked for possible explanations for this finding. One explanation is that there is an uneven distribution of early recurrences during or shortly after discontinuation of anticoagulation.

These recurrences might be explained by other risk factors (such as cancer) or may be the result of a rebound phenomenon[14]. In fact, these early recurrences were not associated with basal homocysteine levels (as were the recurrences during follow-up), so the uneven distribution over the various treatment groups could be attributed to chance. When we excluded early recurrences, the overall risk estimate became 0.76, and the effects in the hyper- and normohomocysteinemic group were quite similar. The same occurs after taking the date of anticoagulant cessation as starting point for the survival analysis. Although, these analyses are post-hoc analyses, they support that the overall estimate of 0.84 (95% CI 0.56 to 1.26) is the best summary of the study, despite an initial heterogeneity of effect.

The domain of our trial was idiopathic venous thrombosis. We had very strict inclusion criteria (objectively confirmed first event of proximal deep-vein thrombosis or pulmonary embolism in absence of major risk factors - major surgery, known malignant disease, pregnancy and puerperium or immobility for more than three weeks -, are aged between 20 to 80 years at time of diagnosis and without obligatory use of B-vitamins). Most of the patients were not eligible because thrombosis occurred after surgery, patients were older than 80, had cancer or had a recurrent event. For these reasons many patients had to be screened in order to include the required number of patients for this study.

An important point in clinical trials with B-vitamins is the difference achieved in homocysteine concentration in the vitamin group and the placebo group. This difference was small in a trial in stroke patients in North-America[15]. In the design of our study we opted for a strong homocysteine lowering effect, which

was found in a schedule with 5 mg folate, 0.4 mg vitamin B12 and 50 mg vitamin B6 [5]

Furthermore our study was done in an area without food-fortification with folate. Therefore, a strong difference in median homocysteine between high-dose multivitamin and placebo of 6.3 µmol/l (42%) in the hyperhomocysteinemic group and 2.9 µmol/l (30%) in the normohomocysteinemic group was found.

An on-treatment analysis based on the percentage of reduction showed a trend to a risk reduction in subjects with the highest reduction in homocysteine. This finding stresses the importance of adequate homocysteine reduction in clinical trials with B-vitamins. The dose-response relationship gives also some indication that our trial does not completely exclude an effect of vitamin supplementation to prevent recurrent venous thrombosis.

Our study was designed in 1995. For the sample size calculation we assumed a risk reduction of 50% that was based on earlier case-control studies and especially on a cohort study in patients with first time venous thrombosis with a relative risk of 2.7 for recurrent thrombosis in patients in the top-quartile of the homocysteine distribution[13]. Findings from others, after the start of this trial, indicated less strong effects of hyperhomocysteinemia on the risk of first thrombosis. In a recent metaanalysis we found a relative risk for venous thrombosis between 1.27 in prospective and 1.60 in retrospective studies for a 5 µmol/l increase in homocysteine[1]. On the basis of a meta-analysis of MTHFR 677TT genotype the risk associated with a 3 µmol/l increase in homocysteine levels was 16% (1,3). So, the main conclusion of our study is that vitamin supplementation for treatment of hyperhomocysteinemia does not results in an apparent decrease in incidence of recurrent events. A second conclusion is that our study has not enough power to detect or rule out a modest risk reduction of 10-20% that is expected now on the base of prospective and genetic studies. However, the question is whether such a modest risk reduction is clinically relevant, because the associated numbers needed to treat are large (75-150 /year) In the field of arterial vascular disease 12 studies on the effect of vitamin treatment on vascular disease are initiated[16] of which three are published now[15,17,18]. None of these trials did show a beneficial effect of vitamin supplementation on the incidence of recurrent vascular events. It should be noted that in these trials vitamin supplementation was added to standard treatment that included generally platelet aggregation inhibitors, cholesterol-lowering drugs and antihypertensive medication, which is not a standard treatment after an event of venous thrombosis. Therefore the effect of vitamin supplementation might be different in a trial in patients with venous thrombosis compared to trials in cardiovascular patients.

Our study was a secondary prevention study. This implies that the risk of recurrent venous thrombosis was the subject of study, which might be different from the risks of first-time venous thrombosis. This is clearly demonstrated by

the observation that factor V Leiden - which is a strong risk factor for first-time venous thrombosis – is not or only weakly associated with recurrent venous thrombosis[19]. Two prospective studies has been published on the risk for a recurrent event of venous thrombosis associated with hyperhomocysteinemia. In the first study elevated homocysteine levels (above the 75the percentile) were associated with a 2.7-fold increase in risk[13]. In the second study no increased risk was found (hazard ratio 0.9 (95% CI 0.5 to 1.6))[19]. In our study baseline homocysteine concentration is a predictor of recurrent venous thrombosis. However, the relative risk is lower than the risk for first time venous thrombosis[1].

One of the problems with secondary prevention studies in venous thrombosis is the diagnosis of a recurrent event. It could be difficult to distinguish between a recurrent event and the persistence of a residual thrombus (especially in DVT). To facilitate uniform diagnosis within the study we did repeated ultrasound examinations after the first event and provided a 'patient passport' with information for the treating physician. There was however no central validation of the diagnosis, which is a potential limitation of the study. We have chosen for the decision of the treating physician to restart anticoagulant treatment as defined endpoint, which is in fact the most clinical relevant parameter.

Several explanations can be given to the observation that baseline homocysteine levels are predictive for a recurrent event but homocysteine lowering does not result in a decrease in incidence. First, it might be a matter of insufficient power of our study to detect small effects. This explanation is supported by the post-hoc analyses and the dose-response relationship. Second, it can be explained by another factor that is related to homocysteine but is not affected by vitamin supplementation. However, based on current knowledge there is no evidence to treat patients with venous thrombosis with B-vitamins in order to prevent recurrent events.

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Chapter 9

General discussion

General discussion

In the studies described in this thesis we address several aspects of hyperhomocysteinemia in relation to venous thrombosis.
After the relationship of elevated homocysteine concentrations and venous thrombosis was established by our research group and others in earlier studies a main issue is whether lowering of homocysteine concentration might prevent recurrence of venous thrombosis (homocysteine can be lowered by the treatment with B vitamins, including vitamin B11 (Folic acid), Vitamin B12 and Vitamin B6).
This question is of interest for two reasons. The first reason is to gather more insight into the pathophysiology of homocysteine metabolism and the possible causal relationship of homocysteine and venous thrombosis. Because laboratory- and animal studies have not yet found a definite mechanism of action, it is of interest to further elucidate the relationship with data from epidemiological, intervention studies. In a randomized trial like ours where homocysteine-lowering therapy by B-vitamins was used to study if lowering homocysteine values does reduce the incidence of venous thrombosis, such an effect would strongly support the causal relationship. The second reason is obvious: reducing the incidence of venous thrombosis would benefit the patients. The outline of the study named **VITRO** (**VI**tamins and Th**RO**mbosis) is described in chapter 2.
Before we could start the VITRO trial we had to find a suitable collection medium for blood samples for homocysteine determination. The determination of homocysteine in EDTA tubes is known to pose problems to epidemiological field studies since homocysteine values rise if the samples are not placed onto ice immediately. Also in clinical practice the assay may lead to false results as a consequence of blood handling errors. We showed that tubes containing acidic citrate are a suitable collection medium for determination of homocysteine concentrations (chapter 3). This observation made it possible for us to use this tube for the intervention study described in chapter 2 and 8, where it was necessary to screen a large group of persons (>4000) with venous thrombosis, under circumstances where immediate processing of samples was not possible. Furthermore we showed that the concentrations measured in the acidic citrate tubes highly correlate to the concentrations measured in EDTA blood. However, reference values need to be established in each laboratory since basic concentrations differ in EDTA blood in comparison to acidic citrate blood (Chapter 4). By performing these studies we became aware of the lack of standardization procedures in homocysteine determination. This implies that homocysteine concentrations or cut-off points from studies should be evaluated and compared with caution. Comparison with

concentrations measured in other studies or even in individual patients is therefore often not possible.
The study described in chapter 5 deals with the effect of oral anticoagulants on homocysteine concentrations. We found no relevant effect of oral anticoagulants on homocysteine concentrations. Therefore for the interpretation of research studies and in individual patients with thrombosis where patients were on anticoagulant at the time of homocysteine measurement this treatment does not have to be taken into account.
When hyperhomocysteinemia is a cause of venous thrombosis, one expects to find an association between homocysteine and components of the clotting system. For this reason we studied the endogenous thrombin potential (ETP) to seek for an association between the ETP and homocysteine values. The ETP is a method to measure the potential to generate thrombin, which is a crucial component of the clotting cascade. In patients with inherited risk factors for venous thrombosis it has been demonstrated that the ETP might be elevated. Therefore we reasoned that when high plasma homocysteine values induce thrombosis this phenomenon might be reflected by elevated ETP values. However, we did not see any association between the ETP and homocysteine levels (Chapter 5).
Venous thrombosis is common in the elderly. The incidence rises from 25 / 100,000 / year at the age of 25 to 500 / 100,000 / year over the age of 80. Since plasma homocysteine levels increase exponentially with age, homocysteine might play an important role in the development of venous thrombosis in this age group. Two previous studies have reported on the risk of hyperhomocysteinemia and the development of venous thrombosis and the relation with age, with conflicting results. We therefore performed a case-control study among elderly patients to evaluate whether elevated homocysteine levels are a risk factor for venous thrombosis in this age group, as described in chapter 7. In this study we found that the homocysteine concentration in plasma is a risk factor for venous thrombosis in elderly individuals, as it is among younger people. Furthermore, we found a graded increase in the risk with increasing homocysteine concentrations. The risk estimate was similar to those reported in studies in younger patient groups. This may imply that the absolute effect of hyperhomocysteinemia is greater among the elderly, because the incidence of thrombosis is much higher.
Finally the results of the VITRO trial are described in chapter 8.
We observed a small effect (15% reduction of venous thrombosis) which did not reach statistical significance. However, the sample size of our study was not large enough to rule out a modest beneficial effect of homocysteine lowering therapy at this level.
Since our study was underpowered for a small effect it cannot answer the question whether homocysteine is of pathophysiological significance in

developing venous thrombosis. In the past years several studies have been published which suggested an association between thrombosis and the MTHFR C677T mutation. Results are however still conflicting since not all studies demonstrate this association. This mutation is associated with higher homocysteine values, especially in those with lower vitamin B11 and B12 levels. Since this mutation is a genetic factor it cannot be influenced by the disease itself or surrounding factors. Though the association between MTHFR C666T and venous thrombosis is at most weak it suggests a causal relation[1-3].
The second reason – but in fact the most relevant one for patients – for designing our study was for clinical purposes. When homocysteine is a cause of thrombosis homocysteine treatment by vitamins was expected to reduce the incidence of recurrent venous thrombosis.
This trial was the first randomized trial that evaluated the effect of vitamin supplementation on venous thrombosis. There have been randomized studies which evaluated vitamin supplementation in arterial thrombosis. One study evaluated the effect of folic acid as prevention for re-stenosis after coronary angioplasty[4,5]. The authors found a clearly beneficial effect of the therapy on the need of revascularization and on composite vascular endpoints (death, non-fatal myocardial infarction and revascularization). A second study, however, found no effect of folic acid on composite endpoints in patients with stable angina pectoris[6]. A third randomized trial compared high versus low dose multivitamin suppletion in the secondary prevention of ischemic stroke, coronary heart disease and death[7]. In this study, no effect of therapy was found on the clinical end-points.
In our study we found no effect, at least not at a statistical significant level. A negative result should always be evaluated in the light of the power of a study. In our case the number of events (recurrent venous thrombosis) was lower than expected, especially in the control arm with high homocysteine levels. This reduced the power.
In our trial we found a non-significant 16% reduction of recurrent events. Therefore we cannot exclude a modest effect, or the absence of any effect. Theoretically a reduction in risk of 25% is to be expected according to estimations by Wald *et al.*[1]. A trial sufficiently powered to demonstrate such an effect would need approximately 4000 patient years in both treatment arms. We do not expect that such a study will be performed. If homocysteine lowering would indeed reduce recurrence by about 15%, the question would be whether this is a clinically relevant reduction that would lead to incorporation of this treatment. The recurrence risk after a first event of venous thrombosis in our study was about 7% per year. A reduction with 15% would imply that about 100 patients would need to receive vitamins to prevent one event of thrombosis per year. In chapter 8 we conclude our research project with the statement that although multivitamin supplementation seems to be safe and is not expensive,

this number needed to treat indicates that vitamin supplementation is not a clinically relevant option in the secondary prevention of venous thrombosis, even in those with hyperhomocysteinemia.

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Samenvatting

Samenvatting

Homocysteine is een aminozuur dat betrokken is bij de methionine stofwisseling. Een verhoogde homocysteïne-concentratie in het bloed heeft de afgelopen jaren in de belangstelling gestaan als mogelijke oorzaak van arteriëel vaatlijden (hartinfarcten, beroertes, etalagebenen), maar ook als oorzaak van veneuze trombose (trombosebenen en longembolieën). Er zijn verscheidene patiënt-controle onderzoeken verricht waaruit bleek dat het homocysteïne bij patiënten die in het verleden een trombosebeen of een longembolie hadden gehad, vaker verhoogd was dan bij mensen die nooit een veneuze trombose hadden doorgemaakt. Dit heeft geleid tot de gedachte dat een verhoogde homocysteïne-concentratie een oorzaak zou zijn van trombose. Deze gedachte werd bevestigd met enkele prospectieve onderzoeken, waaruit bleek dat ook al vóór de trombose de homocysteïne concentraties in het plasma verhoogd waren.
De resultaten van patiënt-controle onderzoeken niet prospectieve onderzoeken bewijzen echter niet dat een verhoogd homocysteïne de oorzaak van trombose is. Ook geven zij geen antwoord op de vraag of verlaging van het homocysteïnegehalte in het bloed trombose kan voorkomen.
De belangrijkste vraag in dit proefschrift is daarom of homocysteïne-verlagende therapie een tweede trombose voorkomt bij trombosepatienten met een verhoogd homocysteïne. Allereerst om de hypothese dat een verhoogd homocysteïne oorzaak van thrombose is, te verifiëren klopt. Als immers verlaging van het homocysteïne gehalte ook het aantal gevallen van trombose verlaagt, zou dat een belangrijke aanwijzing zijn dat homocysteïne inderdaad bijdraagt aan het ontstaan van trombose. Als tweede – en voor patiënten uiteraard van groter belang – het klinische doel van de studie: als door verlaging van het homocysteïne een recidief trombose voorkomen kan worden, is dat klinisch zeer relevant. Dit is vooral zo omdat iedere trombose een zeker risico op ernstige schade of overlijden met zich brengt, maar ook omdat dan voorkomen kan worden dat mensen langdurig bloedverdunners moeten gebruiken. Deze middelen hebben immers ook bijwerkingen.
De onderzoeksvraag hebben we proberen te beantwoorden met behulp van een gerandomiseerd onderzoek, de VItaminen en TROmbose studie (VITRO) (hoofdstuk 2 en 8). Alvorens het onderzoek te beginnen hebben we eerst onderzoek gedaan naar een geschikt buisje om het bloed - noodzakelijk voor de homocysteïne bepaling - in te bewaren. De logistiek van het onderzoek vereiste een bloedafnamebuis waarin het homocysteïne werd gestabiliseerd. In de normaliter gebruikte buizen waaraan EDTA is toegevoegd, stijgt het homocysteïnegehalte als de buis niet direct wordt gekoeld tot 0ºC. In hoofdstuk 3 wordt beschreven dat een bloedafnamebuis waaraan zure citraat is toegevoegd, geschikt is om bloed af te nemen voor bepaling van het

homocysteïne gehalte. In het onderzoek werd echter een verschil waargenomen tussen de homocysteïne concentratie in bloed dat was afgenomen in een EDTA buis en bloed dat was afgenomen in een buisje met zure citraat. Dit heeft geleid tot het onderzoek dat is beschreven in hoofdstuk 4, waarbij dit verschil is gekwantificeerd. Bij dit onderzoek is met verscheidene meetmethoden het homocysteïne bepaald om te beoordelen of er verschillen werden waargenomen. Dit bleek het geval en we concluderen dat de buis met zure citraat een goed afnamemedium is, maar dat referentiewaarden moeten worden bepaald afhankelijk van de meetmethode die wordt gebruikt.
Diverse onderzoeken laten zien dat een verhoogd homocysteïne gehalte in het bloed gerelateerd is aan trombose. Daarbij is veelal bloed afgenomen na de trombose, waarbij patiënten vaak antistollingsmedicatie gebruikten. Ook voor het bepalen van homocysteïne bij de individuele persoon is de realiteit dat die patiënt veelal bloedverdunners gebruikt ten tijde van de bloedafname. Daarom is het van belang te weten of het gebruik van antistolling de bepaling van het homocysteïne al dan niet beïnvloedt. Dit bleek niet het geval (hoofdstuk 5).
De relatie tussen oorzaak en gevolg is niet opgehelderd als het gaat over een verhoogd homocysteïnegehalte in het bloed en de ontwikkeling van trombose. Zo is er tot op heden nog geen aannemelijk werkingsmechanisme beschreven dat verklaart hoe het homocysteïne in het bloed leidt tot een verhoogde stollingsneiging. In hoofdstuk 6 hebben we onderzocht of een verhoogd homocysteïnegehalte invloed heeft op de endogene trombinepotentieel (ETP). De ETP is een maat voor het vermogen om trombine te genereren. Het trombine is de belangrijkste stof bij de vorming van stolsels. Van patiënten met een verhoogde stollingsneiging op basis van een erfelijke afwijking is beschreven dat de ETP verhoogd is. We redeneerden dat als het homocysteïne leidt tot trombose, dit wellicht zou kunnen worden teruggevonden in een verhoging van de ETP, zoals bij de erfelijke stollingsneigingen. Dit zou een aanwijzing zijn voor een directe invloed van het homocysteïne in het bloed op de stolling. Wij konden echter geen verschil waarnemen in de ETP tussen mensen met een verhoogd en met een normaal homocysteïne-gehalte.
Bij de screening van de deelnemers voor het VITRO onderzoek viel op dat bij veel oudere trombosepatiënten verhoogde homocysteïne-gehaltes werden vastgesteld. Het is bekend dat het homocysteïne, onder andere door een verminderde nierfunctie en een verslechterde vitamine-status, bij oudere mensen stijgt. Omdat trombose vaak voorkomt op hoge leeftijd, hebben we onderzocht of het homocysteïne invloed heeft op het krijgen van een trombose op hoge leeftijd. Dit onderzoek staat beschreven in hoofdstuk 7. In een onderzoek met 426 patiënten uit de VITRO studie en 294 controles uit de algemene bevolking toonden we aan dat ook bij mensen boven de 65 jaar een verhoogd homocysteïnegehalte een risicofactor vormt voor het krijgen van een

trombosebeen of longembolie. Omdat in onze interventieonderzoek – hieronder beschreven – veel ouderen betrokken waren, is dat een belangrijke waarneming.
Tot slot wordt de VITRO studie beschreven. Hierbij kregen patiënten die recent een trombosebeen of een longembolie hadden doorgemaakt door loting vitaminen B of een placebo (een pil zonder werkzame stof) toegewezen. Er werden twee groepen patiënten geselecteerd, nl. patiënten met een verhoogd homocysteïne gehalte in het bloed en patiënten met een normale homocysteïne waarde. De patiënten hebben gedurende 2,5 jaar de onderzoeksmedicatie gebruikt zonder te weten of zij vitamine B of placebo slikten. Nadat het onderzoek was afgerond, is bekeken in welke groep de meeste recidief trombosen waren voorgekomen. Het bleek dat in de groep met hoge homocysteïne waarden bij het begin van het onderzoek, vitamine B het homocysteïne weliswaar deed dalen maar het risico op trombose niet verminderde: er was zelfs een stijging van het risico van 13% (hetgeen overigens niet meer is dan door toevalsvariatie zou kunnen zijn opgetreden). In de groep patiënten met normale homocysteïnegehaltes bij het begin van het onderzoek daalde het homocysteïne eveneens en werd een risicoreductie van 35% op trombose gevonden (eveneens binnen de foutmarges van het onderzoek). Voor de totale groep was er een risicoreductie van 16%: 12,2% recidief-trombose bij vitamine gebruik en 14,3% recidief-trombose bij placebo gebruik. De statistische onzekerheid (uitgedrukt in betrouwbaarheids-intervallen) van deze risicoschattingen was echter groot, zodat dit onderzoek niet definitief kan aantonen of uitsluiten of vitamine therapie helpt. Onderzoeken die zijn gepubliceerd in de jaren dat ons onderzoek werd verricht, hebben duidelijk gemaakt dat het aantal patiënten dat was geselecteerd in de VITRO studie te klein om een klein effect van therapie aan te tonen, dat op theoretische gronden niet meer zou kunnen zijn dan een risicoreductie van 15 tot 20%. Wij zijn bij het begin van onze onderzoek uitgegaan van een groter verschil. Nieuw onderzoek met meer patiënten (4000) zijn vereist om onze onderzoeksvraag definitief te beantwoorden. Ons onderzoek heeft aangetoond dat vitaminetherapie zeker geen groot effect heeft op het optreden van recidieven. Gegeven de kans op een recidief trombose (ongeveer 7% per jaar) betekent dit dat zelfs wanneer een reductie van ongeveer 15% behaald zou kunnen worden met vitamines, 100 mensen een jaar behandeld moeten worden om een geval van trombose te voorkomen. Dat betekent dat homocysteïne verlagende therapie onvoldoende zal bijdragen aan het verminderen van het probleem van trombose en dat andere vormen van therapie gerealiseerd zullen moeten worden.

Dankwoord

Dankwoord

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Curriculum vitae

Curriculum vitae

De auteur van dit proefschrift werd op 2 mei 1966 in ‘s Hertogenbosch geboren en woonde tot 1984 in Helvoirt. In 1984 behaalde hij het eindexamen aan het gymnasium “Beekvliet” te Sint Michielsgestel. Dit was tevens het aanvangsjaar van de studie geneeskunde in Utrecht. In 1990 behaalde hij het doctoraalexamen. Voor aanvang van de co-schappen heeft hij neurologisch onderzoek verricht bij Prof. C. Warlow in Edinburgh, Schotland. Het co-schap gynaecologie en obstetrie werd gedaan in het Kalafong Ziekenhuis in Pretoria, Zuid-Afrika. Nadat in 1994 het artsexamen was behaald, is hij als arts-assistent interne geneeskunde werkzaam geweest in het Willem-Alexander Ziekenhuis in ’s Hertogenbosch (Dr. P.J. Lestrade). In 1995 werd begonnen met het onderzoek dat heeft geleid tot dit proefschrift bij de afdeling Haematologie van het Leyenburg Ziekenhuis te Den Haag (Dr. W.B.J. Gerrits en Dr. P.W. Wijermans). Vanaf 2000 was hij werkzaam in het Máxima Medisch Centrum te Eindhoven. In 2001 startte hij in dit ziekenhuis met de opleiding interne geneeskunde (Dr. H.R. Haak). In 2004 werd de opleiding voortgezet in het Academisch Ziekenhuis Maastricht (Prof. dr. C.D.A. Stehouwer). In oktober 2006 is hij begonnen met het aandachtsgebied Immunologie en Allergologie (Prof. dr. J.W. Cohen-Tervaert).