Cardiometabolic risk factors and venous thrombosis

Morelli, V.M.

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Vânia Maris Morelli

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Promotores

Prof. dr. S.C. Cannegieter

Prof. dr. F.R. Rosendaal

Co-promotor

Dr. W.M. Lijfering

Leden promotiecommissie

Prof. dr. J.G. van der Bom
Prof. dr. J.A. Lisman (Groningen University Medical Center)
Dr. M.H.A. Bos
Dr. S.K. Brækkan (University of Tromsø, Norway)

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TABLE OF CONTENTS

Chapter 1

General Introduction and Outline of this Thesis

GENERAL INTRODUCTION

Thrombosis

The obstructive clot formation that defines thrombosis is the end product of an imbalance between procoagulant, anticoagulant and fibrinolytic factors [1]. The obstructive clot formation in arteries most commonly leads to myocardial infarction and ischemic stroke. In the venous system, the blood clot is most often formed in the deep veins of the leg, i.e. deep vein thrombosis. Pulmonary embolism occurs when the blood clot dislodges from its site and embolizes to the arterial blood supply of the lungs.

The current understanding of the pathophysiology of thrombosis dates back to 1856 when the pathologist Rudolf Virchow explained thrombosis as the result of changes in blood flow, damage to the vessel wall, and changes in blood composition [2]. This broad concept is still valid. However, the mechanisms that are at the basis of venous versus arterial thrombosis are different. As depicted in Fig. 1, an arterial thrombus is typically formed after rupture of an atherosclerotic plaque (Fig. 1A), whereas venous thrombi assemble on the surface of a largely intact vessel wall (Fig. 1B) [3].

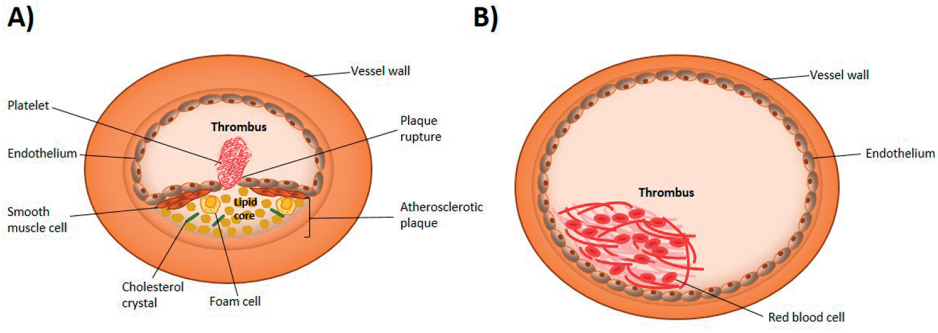

Figure 1. Triggers of arterial and venous thrombosis. A) Artery. The primary trigger of arterial thrombosis is rupture of an atherosclerotic plaque. This involves disruption of the endothelium and release of constituents of the plaque into the lumen of the blood vessel [3]. B) Vein. By contrast, in venous thrombosis, the endothelium remains intact but can be converted from a surface with anticoagulant properties to one with procoagulant properties. Venous thrombosis can be triggered by several factors: abnormal blood flow (stasis), altered properties of the blood itself with increased coagulability and alterations in the endothelium [3].

Venous thrombosis

Venous thrombosis, encompassing deep vein thrombosis and pulmonary embolism, is a common disease, with an overall incidence of 1-2 per 1000 persons each year [4]. Incidence rates rise exponentially with age, from < 0.005% per year in children to nearly 0.5% per year in the elderly [5]. Venous thrombosis is the third most common cardiovascular disease (CVD) after myocardial infarction and stroke, and has become a major challenge to health care systems due to frequent hospitalizations, severe co-morbidities, and a high mortality rate [6-8]. The economic burden caused by long-term complications of venous thrombosis is also a major concern to public health. In individuals who survive a first event of venous thrombosis, the risk of recurrence is high, with a 5-year cumulative incidence ranging from 12% to up to 30% [8-11]. On the basis of contemporary prospective studies with 12 months or longer follow-up, one-third to one-half of deep vein thrombosis patients can expect to develop post-thrombotic syndrome, a potentially debilitating condition for which patients frequently seek medical advice [12]. Even though the incidence of chronic thromboembolic pulmonary hypertension is low in survivors of acute pulmonary embolism (~3%), this complication is associated with poor prognosis when surgical removal of the chronic thrombi is not feasible [13].

Venous thrombosis is a multicausal disease occurring as the result of interacting genetic, environmental and behavioral risk factors [1]. Despite an extensive list on risk factors, there are continuing efforts to identify novel and clinically relevant risk factors for a first and recurrent venous thrombosis. Knowledge of risk factors is crucial, as it may guide strategies to prevent and treat venous thrombosis, thereby allowing improvement of patient care.

Several studies, mainly in the past decade, have shown a relationship between venous thrombosis and arterial CVD [14-19], and suggested that venous thrombosis and arterial CVD may be two different phenotypes of the same disease [20]. Indeed, cardiometabolic risk factors, such as dyslipidemia, inflammation and obesity, are well known to increase the risk of arterial CVD but may also be associated with venous thrombosis [20,21]. Next the rationale behind the association between venous thrombosis and arterial CVD is addressed in more detail.

Association between venous thrombosis and arterial CVD

Arterial CVD (i.e. myocardial infarction and ischemic stroke) and venous thrombosis have traditionally been regarded as two separate diseases due to different pathophysiology (Fig. 1), clinical presentation, and treatment [22]. In the past decade, however, this notion has been challenged. Several studies have shown that venous thrombosis patients have an increased risk of subsequent arterial CVD [15-19]. In a large population-based cohort study in Denmark, patients with venous thrombosis

had an about 2-fold increased risk of subsequent arterial cardiovascular events compared with population controls, and the risk, albeit attenuated, persisted over the long-term follow-up [15]. One of the hypotheses for the association between venous thrombosis and arterial CVD is that the two diseases may share common risk factors [20]. In line with this hypothesis, the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) follow-up study also showed that patients with venous thrombosis had a 2.2-fold increased risk of subsequent arterial cardiovascular events compared with controls from the community [17]. However, upon adjustment for potential common risk factors (confounders), i.e. factors that are related to both exposure (venous thrombosis) and outcome (arterial CVD), but do not lie on the causal pathway between exposure and outcome (see Fig. 2), the risk estimate was attenuated to 1.5. These results suggest that the increased risk of arterial CVD in venous thrombosis patients could be explained, at least in part, by common risk factors.

Among the traditional cardiometabolic risk factors, obesity has consistently been associated with venous thrombosis [23-26]. However, the relationship between other traditional cardiometabolic risk factors and venous thrombosis is less clear. For instance, dyslipidemia, high glucose levels, and renal dysfunction are established cardiometabolic risk factors [27-29]. Still, whether the above mentioned factors are associated with risk of a first or recurrent venous thrombosis is not known in detail due to controversial or scarce data in literature. In this thesis, the association between lipid levels and risk of a first and recurrent venous thrombosis is studied in detail in **chapters 2** and **3,** respectively. The associations of renal dysfunction and glucose levels with recurrent venous thrombosis are further addressed in **chapter 4**. Some

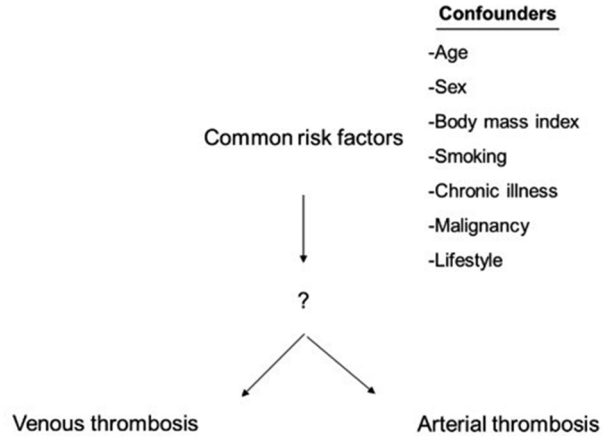

Figure 2. Proposed mechanism for the association between venous thrombosis and an increased risk of subsequent arterial cardiovascular disease [17,20].

hematologic variables, such as red cell distribution width, that are associated with risk of arterial CVD [30] or first venous thrombosis [31-35], are additionally studied in chapter 4 in relation to recurrence.

Knowledge on how cardiometabolic risk factors are related to components of the hemostatic system may provide insights on possible mechanisms underlying the associations of these factors with venous thrombosis. In this thesis, the relationship of lipids, particularly serum and hepatic triglycerides, with hemostatic factors is assessed in **chapters 5** and **6**.

The aim of this thesis is to investigate the associations of traditional cardiometabolic risk factors with risk of a first and recurrent venous thrombosis. Additionally, associations between lipids and levels of several hemostatic factors are assessed.

Study population

To answer the research questions addressed in the chapters of this thesis, data from different population-based studies are used: the MEGA study, the MEGA follow-up study, and the Netherlands Epidemiology of Obesity (NEO) study. These studies will be briefly described.

The MEGA study

The MEGA study is a large case-control study into risk factors for venous thrombosis [36]. Between March 1999 and September 2004, 4956 consecutive patients aged 18-70 years with a first objectively confirmed deep vein thrombosis of the leg or pulmonary embolism were enrolled from six anticoagulation clinics in the Netherlands. For this thesis, participants with active or a previous history of malignancy within 5 years before the index date are excluded. Among the 4956 patients, 4463 are eligible for the studies that are performed in this thesis. Control subjects, without a history of venous thrombosis, are partners of the patients (n= 3297) or individuals approached by random digit dialing [RDD] (n= 3000). The exclusion of control subjects with active or a previous history of malignancy results in 3222 partner and 2939 RDD controls.

All participants filled in a detailed questionnaire on their medical history and the presence of possible risk factors for venous thrombosis. Additionally, blood was collected from patients three months after discontinuation of anticoagulant treatment or one year after the event if they continued anticoagulant treatment for more than one year. Partner controls provided blood at the same time as the patient, and RDD controls provided blood within a few weeks after the questionnaire was sent. This study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants.

The MEGA follow-up study

The aim of the MEGA follow-up study is to assess the incidence of recurrent events and to identify new risk factors and predictors of recurrences [37]. Of 4956 patients included in the MEGA study, 225 did not consent for follow-up, leaving 4731 patients for the MEGA follow-up study. As patients with active or previous history of malignancy within 5 years before the first event of venous thrombosis are excluded from this thesis, there are 4275 consenting patients eligible for follow-up.

Between June 2008 and July 2009, patients were asked whether they had developed a recurrent venous thrombotic event by means of a short answer form. Furthermore, between 2007 and 2009, the vital status of all MEGA follow-up patients was obtained from the Dutch population register and causes of death from the national registry of death certificates. Data from the answering forms, causes of death, anticoagulation clinics and discharge letters from treating physicians were combined to make a classification of certain and uncertain recurrences [37].

The NEO study

The NEO study is a population-based cohort study designed to investigate pathways that lead to obesity-related diseases [38]. The NEO study includes 6671 participants, with an oversampling of individuals with overweight or obesity. Between September 2008 and September 2012, men and women aged 45-65 years with a self-reported body mass index (BMI) of 27 kg/m^2 or higher living in the greater area of Leiden (in the West of The Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged 45-65 years from one municipality (Leiderdorp) were invited to participate, irrespective of their BMI, in order to obtain a reference distribution of BMI.

All participants visited the NEO study centre after an overnight fast for baseline measurements, including blood sampling and anthropometry. Prior to the baseline visit, participants completed questionnaires on demographic, lifestyle and clinical data. In addition, among the eligible participants, 2580 were randomly selected to undergo localized hydrogen 1 (^{1}H) magnetic resonance spectroscopy to assess hepatic triglyceride content, and magnetic resonance imaging to assess abdominal subcutaneous and visceral fat. This study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants.

OUTLINE OF THIS THESIS

In **chapter 2**, the association between lipid levels and risk of a first venous thrombosis is investigated in the MEGA study, and the possible underlying mechanism is evaluated in detail, considering confounding by common risk factors and mediation via hemostatic factors and C-reactive protein. An important rationale supporting the research question addressed in this chapter is the fact that several studies have shown that lipid-lowering drugs (statins, most notably rosuvastatin) are associated with a decreased risk of venous thrombosis [39-41], thereby indicating a possible role for lipids in the pathophysiology of venous thrombosis. Lipids are also interesting candidates to be investigated in relation to the risk of recurrent venous thrombosis, as statins have been associated with a decreased risk of recurrence [42-44]. Therefore, in **chapter 3,** the association between lipid levels and risk of recurrent venous thrombosis is assessed in the MEGA follow-up study.

Kidney function, measured as estimated glomerular filtration rate (eGFR), glucose levels, and hematologic variables (i.e., cell blood count) are easily obtainable tests, not influenced by anticoagulation on their measurements, that have been associated with risk of a first venous thrombosis in several studies [31-35,45-51]. However, data on the role of these tests in assessing risk of recurrent venous thrombosis are scarce for hematologic variables [33,52] and eGFR [53], or even unknown for glucose levels. **Chapter 4** describes the association of eGFR, glucose levels and hematologic variables with recurrent venous thrombosis.

Previous studies have shown that hemostatic factor levels are interrelated and clustered together [54-56]. However, results were not consistent, probably due to differences in samples sizes, the study population and the hemostatic factors studied. Furthermore, since venous thrombosis and arterial CVD have been shown to be associated [14-19], and may share some traditional cardiometabolic risk factors [20,21], in **chapter 5** we hypothesize that hemostatic factors cluster with lipids and C-reactive protein (i.e. an inflammatory risk marker of vascular disease) [57].

Excess fat accumulation in the liver, also referred to as non-alcoholic fatty liver disease (NAFLD), is strongly associated with obesity [58], and could be one of the mechanisms by which obesity increases the risk of venous thrombosis. In the past two decades, several small studies have investigated the association between NAFLD and hemostatic factors [59-67]. However, whether NAFLD contributes to levels of coagulation factors beyond total body and visceral fat is unclear, particularly in relation to those factors associated with an increased risk of venous thrombosis, i.e. factors VIII, IX and XI [1]. In **chapter 6**, the association between liver fat, quantified as hepatic triglyceride content by magnetic resonance spectroscopy, and levels of factors VIII, IX and XI, and fibrinogen is investigated in the NEO study while adjusting for total body and visceral fat.

REFERENCES

1. Rosendaal FR. Venous thrombosis: the role of genes, environment, and behavior. Hematol Am Soc Hematol Educ Progr 2005; 1-12.
2. Virchow R. Phlogose und Trombose im Gefäßsystem: Gesammelte Abhandlungen zur Wissenschaftlichen Medizin. Frankfurt Staatsdruckerei 1856.
3. Mackman N. Triggers, targets and treatments for thrombosis. Nature 2008; 451: 914-8.
4. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 2007; 5: 692-9.
5. White RH. The epidemiology of venous thromboembolism. Circulation 2003; 107: I4-8.
6. Raskob GE, Angchaisuksiri P, Blanco AN, Buller H, Gallus A, Hunt BJ, Hylek EM, Kakkar A, Konstantinides SV, McCumber M, Ozaki Y, Wendelboe A, Weitz JI; ISTH Steering Committee for World Thrombosis Day. Thrombosis: a major contributor to global disease burden. Arterioscler Thromb Vasc Biol 2014; 34: 2363-71.
7. Rosendaal FR, Raskob GE. On World Thrombosis Day. Lancet 2014; 384: 1653-4.
8. Arshad N, Bjøri E, Hindberg K, Isaksen T, Hansen JB, Braekkan SK. Recurrence and mortality after first venous thromboembolism in a large population-based cohort. J Thromb Haemost 2017; 15: 295-303.
9. Christiansen SC, Cannegieter SC, Koster T, Vandenbroucke JP, Rosendaal FR. Thrombophilia, clinical factors, and recurrent venous thrombotic events. JAMA 2005; 293: 2352-61.
10. Hansson PO, Sörbo J, Eriksson H. Recurrent venous thromboembolism after deep vein thrombosis: incidence and risk factors. Arch Intern Med 2000; 160: 769-74.
11. Prandoni P, Noventa F, Ghirarduzzi A, Pengo V, Bernardi E, Pesavento R, Iotti M, Tormene D, Simioni P, Pagnan A. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. Haematologica 2007; 92: 199-205.
12. Kahn SR. The post-thrombotic syndrome. Hematology Am Soc Hematol Educ Program 2010: 216-20.
13. Ende-Verhaar YM, Cannegieter SC, Vonk Noordegraaf A, Delcroix M, Pruszczyk P, Mairuhu AT, Huisman MV, Klok FA. Incidence of chronic thromboembolic pulmonary hypertension after acute pulmonary embolism: a contemporary view of the published literature. Eur Respir J 2017; 49(2).
14. Prandoni P, Bilora F, Marchiori A, Bernardi E, Petrobelli F, Lensing AW, Prins MH, Girolami A. An association between atherosclerosis and venous thrombosis. N Engl J Med 2003; 348: 1435-41.
15. Sørensen HT, Horvath-Puho E, Pedersen L, Baron JA, Prandoni P. Venous thromboembolism and subsequent hospitalisation due to acute arterial cardiovascular events: a 20-year cohort study. Lancet 2007; 370: 1773-9.

16. Becattini C, Vedovati MC, Ageno W, Dentali F, Agnelli G. Incidence of arterial cardiovascular events after venous thromboembolism: a systematic review and a meta-analysis. J Thromb Haemost 2010; 8: 891-7.

17. Roach RE, Lijfering WM, Flinterman LE, Rosendaal FR, Cannegieter SC. Increased risk of CVD after VT is determined by common etiologic factors. Blood 2013; 121: 4948-54.

18. Lind C, Flinterman LE, Enga KF, Severinsen MT, Kristensen SR, Braekkan SK, Mathiesen EB, Njølstad I, Cannegieter SC, Overvad K, Hansen JB. Impact of incident venous thromboembolism on risk of arterial thrombotic diseases. Circulation 2014; 129: 855-63.

19. Ljungqvist M, Holmström M, Kieler H, Odeberg J, Lärfars G. Cardiovascular disease and mortality after a first episode of venous thromboembolism in young and middle-aged women. Thromb Res 2016; 138: 80-5.

20. Lijfering WM, Flinterman LE, Vandenbroucke JP, Rosendaal FR, Cannegieter SC. Relationship between venous and arterial thrombosis: a review of the literature from a causal perspective. Semin Thromb Hemost 2011; 37: 885-96.

21. Ageno W, Becattini C, Brighton T, Selby R, Kamphuisen PW. Cardiovascular risk factors and venous thromboembolism: a meta-analysis. Circulation 2008; 117: 93-102.

22. Fauci AS, Braunwald E, Kasper DL, et al. Harrison's Principles of Internal Medicine. 17th ed. New York, NY: McGraw-Hill; 2008.

23. Abdollahi M, Cushman M, Rosendaal FR. Obesity: risk of venous thrombosis and the interaction with coagulation factor levels and oral contraceptive use. Thromb Haemost 2003; 89: 493-48.

24. Severinsen MT, Kristensen SR, Johnsen SP, Dethlefsen C, Tjønneland A, Overvad K. Anthropometry, body fat, and venous thromboembolism: a Danish follow-up study. Circulation 2009; 120: 1850-7.

25. Borch KH, Braekkan SK, Mathiesen EB, Njølstad I, Wilsgaard T, Størmer J, Hansen JB. Anthropometric measures of obesity and risk of venous thromboembolism: the Tromso study. Arterioscler Thromb Vasc Biol 2010; 30: 121-7.

26. Braekkan SK, Siegerink B, Lijfering WM, Hansen JB, Cannegieter SC, Rosendaal FR. Role of obesity in the etiology of deep vein thrombosis and pulmonary embolism: current epidemiological insights. Semin Thromb Hemost 2013; 39: 533-40.

27. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. JAMA 2009; 302: 1993-2000.

28. Laakso M. Hyperglycemia and cardiovascular disease in type 2 diabetes. Diabetes 1999; 48: 937-42.

29. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 2004; 351: 1296-305.

30. Zalawadiya SK, Veeranna V, Niraj A, Pradhan J, Afonso L. Red cell distribution width and risk of coronary heart disease events. Am J Cardiol 2010; 106: 988-93.

31. Cay N, Unal O, Kartal MG, Ozdemir M, Tola M. Increased level of red blood cell distribution width is associated with deep venous thrombosis. Blood Coagul Fibrinolysis 2013; 24: 727-31.

32. Zöller B, Melander O, Svensson P, Engström G. Red cell distribution width and risk for venous thromboembolism: a population-based cohort study. Thromb Res 2014; 133: 334-9.

33. Ellingsen TS, Lappegård J, Skjelbakken T, Brækkan SK, Hansen JB. Red cell distribution width is associated with incident venous thromboembolism (VTE) and case-fatality after VTE in a general population. Thromb Haemost 2015; 113: 193-200.

34. Bucciarelli P, Maino A, Felicetta I, Abbattista M, Passamonti SM, Artoni A, Martinelli I. Association between red cell distribution width and risk of venous thromboembolism. Thromb Res 2015; 136: 590-4.

35. Rezende SM, Lijfering WM, Rosendaal FR, Cannegieter SC. Hematologic variables and venous thrombosis: red cell distribution width and blood monocyte count are associated with an increased risk. Haematologica 2014; 99: 194-200.

36. van Stralen KJ, Rosendaal FR, Doggen CJ. Minor injuries as a risk factor for venous thrombosis. Arch Intern Med 2008; 168: 21-6.

37. Timp JF, Lijfering WM, Flinterman LE, van Hylckama Vlieg A, le Cessie S, Rosendaal FR, Cannegieter SC. Predictive value of factor VIII levels for recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2015; 13: 1823–32.

38. de Mutsert R, den Heijer M, Rabelink TJ, Smit JW, Romijn JA, Jukema JW, de Roos A, Cobbaert CM, Kloppenburg M, le Cessie S, Middeldorp S, Rosendaal FR. The Netherlands Epidemiology of Obesity (NEO) study: study design and data collection. Eur J Epidemiol 2013; 28: 513-523.

39. Glynn RJ, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT, Ridker PM. A randomized trial of rosuvastatin in the prevention of venous thromboembolism. N Engl J Med 2009; 360: 1851-61.

40. Ramcharan AS, Van Stralen KJ, Snoep JD, Mantel-Teeuwisse AK, Rosendaal FR, Doggen CJ. HMG-CoA reductase inhibitors, other lipid-lowering medication, antiplatelet therapy, and the risk of venous thrombosis. J Thromb Haemost 2009; 7: 514-20.

41. Rahimi K, Bhala N, Kamphuisen P, Emberson J, Biere-Rafi S, Krane V, Robertson M, Wikstrand J, McMurray J. Effect of statins on venous thromboembolic events: a meta-analysis of published and unpublished evidence from randomised controlled trials. PLoS Med 2012; 9: e1001310.

42. Biere-Rafi S, Hutten BA, Squizzato A, Ageno W, Souverein PC, de Boer A, Gerdes VE, Büller HR, Kamphuisen PW. Statin treatment and the risk of recurrent pulmonary embolism. Eur Heart J 2013; 34: 1800-6.

43. Nguyen CD, Andersson C, Jensen TB, Gjesing A, Schjerning Olsen AM, Malta Hansen C, Büller H, Torp-Pedersen C, Gislason GH. Statin treatment and risk of recurrent venous thromboembolism: a nationwide cohort study. BMJ Open 2013; 3: e003135.

44. Schmidt M, Cannegieter SC, Johannesdottir SA, Dekkers OM, Horváth-Puhó E, Sørensen HT. Statin use and venous thromboembolism recurrence: a combined nationwide cohort and nested case-control study. J Thromb Haemost 2014; 12: 1207-15.

45. Ocak G, Vossen CY, Lijfering WM, Verduijn M, Dekker FW, Rosendaal FR, Cannegieter SC. Role of hemostatic factors on the risk of venous thrombosis in people with impaired kidney function. Circulation 2014; 129: 683-91.

46. Mahmoodi BK, Gansevoort RT, Næss IA, Lutsey PL, Brækkan SK, Veeger NJ, Brodin EE, Meijer K, Sang Y, Matsushita K, Hallan SI, Hammerstrøm J, Cannegieter SC, Astor BC, Coresh J, Folsom AR, Hansen JB, Cushman M. Association of mild to moderate chronic kidney disease with venous thromboembolism: pooled analysis of five prospective general population cohorts. Circulation 2012; 126: 1964-71.

47. Cheung KL, Zakai NA, Folsom AR, Kurella Tamura M, Peralta CA, Judd SE, Callas PW, Cushman M. Measures of Kidney Disease and the Risk of Venous Thromboembolism in the REGARDS (Reasons for Geographic and Racial Differences in Stroke) Study. Am J Kidney Dis 2017; 70: 182-190.

48. Hermanides J, Cohn DM, Devries JH, Kamphuisen PW, Huijgen R, Meijers JC, Hoekstra JB, Büller HR. Venous thrombosis is associated with hyperglycemia at diagnosis: a case-control study. J Thromb Haemost 2009; 7: 945-9.

49. Tichelaar YIGV, Lijfering WM, ter Maaten JC, Kluin-Nelemans JC, Meijer K. High levels of glucose at time of diagnosing venous thrombosis: a case-control study. J Thromb Haemost 2011; 9: 883-5.

50. Braekkan SK, Mathiesen EB, Njølstad I, Wilsgaard T, Hansen JB. Hematocrit and risk of venous thromboembolism in a general population. The Tromso study. Haematologica 2010; 95: 270-5.

51. Basavaraj MG, Brækkan SK, Brodin E, Østerud B, Hansen JB. Monocyte count and procoagulant functions are associated with risk of venous thromboembolism: the Tromsø study. J Thromb Haemost 2011; 9: 1673-6.

52. Eischer L, Tscholl V, Heinze G, Traby L, Kyrle PA, Eichinger S. Hematocrit and the risk of recurrent venous thrombosis: a prospective cohort study. PLoS One 2012; 7: e38705.

53. Parikh AM, Spencer FA, Lessard D, Emery C, Baylin A, Linkletter C, Goldberg RJ. Venous thromboembolism in patients with reduced estimated GFR: a population-based perspective. Am J Kidney Dis 2011; 58: 746-55.

54. Sakkinen PA, Wahl P, Cushman M, Lewis MR, Tracy RP. Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome. Am J Epidemiol 2000; 152: 897-907.

55. Van Hylckama Vlieg A, Callas PW, Cushman M, Bertina RM, Rosendaal FR. Inter-relation of coagulation factors and d-dimer levels in healthy individuals. J Thromb Haemost 2003; 1: 516-22.

56. Vossen CY, Callas PW, Hasstedt SJ, Long GL, Rosendaal FR, Bovill EG. A genetic basis for the interrelation of coagulation factors. J Thromb Haemost 2007; 5: 1930-5.

57. Doggen CJ, Berckmans RJ, Sturk A, Manger Cats V, Rosendaal FR. C-reactive protein, cardiovascular risk factors and the association with myocardial infarction in men. J Intern Med 2000; 248: 406-14.

58. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science 2011; 332: 1519-1523.

59. Cigolini M, Targher G, Agostino G, Tonoli M, Muggeo M, De Sandre G. Liver steatosis and its relation to plasma haemostatic factors in apparently healthy men-role of the metabolic syndrome. Thromb Haemost 1996; 76: 69-73.

60. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. Diabet Med 2005; 22: 1354-58.

61. Targher G, Bertolini L, Rodella S, Lippi G, Franchini M, Zoppini G, Muggeo M, Day CP. NASH predicts plasma inflammatory biomarkers independently of visceral fat in men. Obesity 2008; 16: 1394-99.

62. Kargili A, Cipil H, Karakurt F, Kasapoglu B, Koca C, Aydin M, Uysal S, Ikizek M, Uz E, Balcik OS, Kosar A. Hemostatic alterations in fatty liver disease. Blood Coagul Fibrinolysis 2010; 21: 325-7.

63. Verrijken A, Francque S, Mertens I, Prawitt J, Caron S, Hubens G, Van Marck E, Staels B, Michielsen P, Van Gaal L. Prothrombotic factors in histologically proven nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology 2014; 59: 121-9.

64. Kotronen A, Joutsi-Korhonen L, Sevastianova K, Bergholm R, Hakkarainen A, Pietiläinen KH, Lundbom N, Rissanen A, Lassila R, Yki-Järvinen H. Increased coagulation factor VIII, IX, XI and XII activities in non-alcoholic fatty liver disease. Liver Int 2011; 31: 176-83.

65. Chang ML, Hsu CM, Tseng JH, Tsou YK, Chen SC, Shiau SS, Yeh CT, Chiu CT. Plasminogen activator inhibitor-1 is independently associated with non-alcoholic fatty liver disease whereas leptin and adiponectin vary between genders. J Gastroenterol Hepatol 2015; 30: 329-36.

66. Tripodi A, Fracanzani AL, Primignani M, Chantarangkul V, Clerici M, Mannucci PM, Peyvandi F, Bertelli C, Valenti L, Fargion S. Procoagulant imbalance in patients with non -alcoholic fatty liver disease. J Hepatol 2014; 61: 148-54.

67. Potze W, Siddiqui MS, Boyett SL, Adelmeijer J, Daita K, Sanyal AJ, Lisman T. Preserved hemostatic status in patients with non-alcoholic fatty liver disease. J Hepatol 2016; 65: 980-987.

Chapter 2

Lipid levels and risk of venous thrombosis: results from the MEGA-study

V.M. Morelli, W.M. Lijfering, M.H.A. Bos, F.R. Rosendaal, S.C. Cannegieter

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ABSTRACT

The relationship between lipid levels and risk of venous thrombosis is not well established. We aimed to assess the association between several lipids and risk of venous thrombosis using data from a population-based case-control study, and to evaluate the underlying mechanism, considering confounding by common risk factors and mediation via hemostatic factors and C-reactive protein. From the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study, 2234 patients with a first venous thrombosis and 2873 controls were included. Percentile categories of total/low-density lipoprotein/high-density lipoprotein cholesterol, triglycerides, and apolipoproteins B and A1 were established in controls (<10th, 10th-25th, 25th-75th [reference], 75th-90th, >90th percentile). In age- and sex-adjusted models, decreasing levels of apolipoproteins B and A1 were dose-dependently associated with increased thrombosis risk, with odds ratios of 1.35 (95% confidence interval, 1.12-1.62) and 1.50 (95% confidence interval, 1.25-1.79) for the lowest category vs. the reference category, respectively. The dose-response relation remained with further adjustment for body mass index, estrogen use, statin use, and diabetes. Although apolipoproteins B and A1 were associated with several hemostatic factors and C-reactive protein, none explained the increased risk in mediation analyses. The other lipids were not associated with venous thrombosis risk. In conclusion, decreasing levels of apolipoproteins B and A1 were associated with increased risk of venous thrombosis. Our findings are consistent with experimental data on the anticoagulant properties of apolipoproteins B and A1. These findings need to be confirmed and the underlying mechanism further investigated.

INTRODUCTION

Venous thrombosis and arterial cardiovascular disease have been traditionally regarded as separate diseases with distinct causes and treatment. However, several studies in the past decade have shown that patients with venous thrombosis (i.e., deep vein thrombosis or pulmonary embolism) have an increased risk of subsequent arterial disease [1]. As lipid levels can be modulated by lifestyle intervention and drug therapy [2], the potential association between lipids and venous thrombosis and the related pathophysiology is a relevant clinical issue worth pursuing. Indeed, previous data have shown that lipid-lowering drugs (statins, most notably rosuvastatin) are associated with a decreased risk of venous thrombosis [3-5], which might indicate a possible role for lipids in the pathophysiology of venous thrombosis. However, whether lipids are associated with venous thrombosis is not known in detail due to controversial results among epidemiological studies [6-13].

The association between lipid levels and venous thrombosis might be explained by common factors that are related to both lipids and risk of venous thrombosis (confounders), such as age [14-16], sex [11,14,15], lifestyle [2,17], body mass index (BMI) [2,15,16], estrogen use [15,16,18], statin use [3-5,19], and diabetes mellitus [2,20] (Figure 1). If causal, the association could be explained by factors that are a consequence of lipid properties on hemostasis [21] and inflammation [22] (mediators in the causal pathway) (Figure 1). These might increase the risk of venous thrombosis and could include changes in levels of hemostatic factors [16] and C-reactive protein (CRP) [10].

Therefore, the aim of our study was to assess the association between lipid levels and risk of venous thrombosis, and to evaluate the underlying mechanism, with particular attention to confounding and mediation via hemostatic factors and CRP. For this purpose, we used data of a large, population-based case-control study on the etiology of venous thrombosis (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis [MEGA] study). In the present analysis, we included the major lipid analytes, i.e., total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides. We also analysed apolipoprotein A1 (apo A1), which is the major protein component of HDL-C [23], and apolipoprotein B (apo B), which is the protein component of the very low-density/low-density lipoprotein spectrum (apo B-containing lipoproteins) [23].

METHODS

Study design

Between March 1999 and September 2004, consecutive patients aged 18-70 years with a first objectively confirmed deep vein thrombosis or pulmonary embolism were

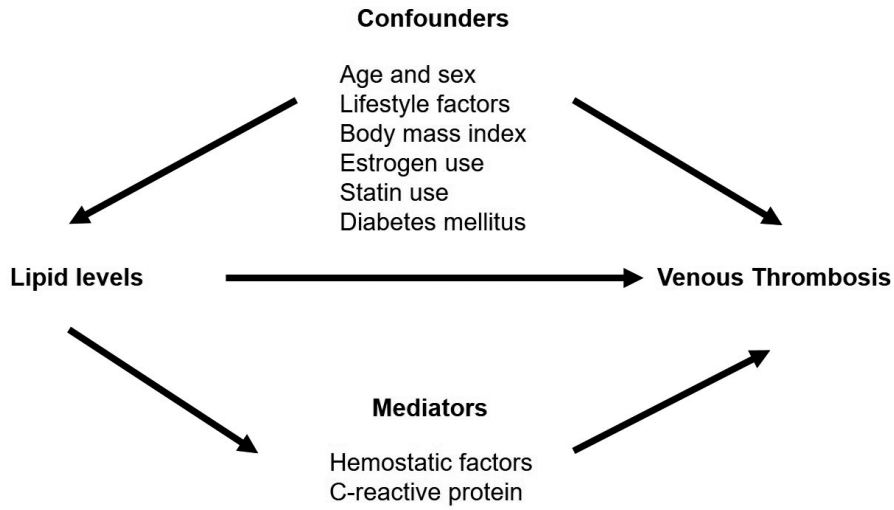

Figure 1. Causal diagram of the association between lipid levels and venous thrombosis

included in the MEGA study from 6 anticoagulation clinics in the Netherlands [24]. For the current analysis, patients with arm vein thrombosis and participants with active or previous history of malignancy within 5 years before the index date, and participants with missing data on malignancy were excluded. In the MEGA study, blood sampling was determined by calendar time, i.e., for logistic reasons participants were asked to provide blood samples up to June 2002 only [24]. Of the 4463 patients eligible for this study, 2237 provided blood samples (Figure 2). Controls were included from 2 sources: partners of patients and subjects reached by random digit dialing (RDD) [24] (Figure 2). Of the 3222 eligible partners (aged 18-70 years, and without venous thrombosis), 1459 provided blood. Of the 2939 eligible RDD controls (frequency matched for age and sex to the patients, and without venous thrombosis), 1422 provided blood. This study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants. The MEGA study has been conducted according to the principles expressed in the Declaration of Helsinki and described in detail elsewhere [24].

Data collection and blood sampling

All participants were asked to complete a questionnaire on many potential risk factors for venous thrombosis [24]. Of interest for this analysis are the items on body weight and height, lifestyle, estrogen- and statin-use, and self-reported diabetes. BMI was calculated by dividing weight (kg) by height squared (m^2). A BMI between 18.5-25 kg/m^2 was defined as normal, between 25-30 kg/m^2 as overweight and >30 kg/m^2 as

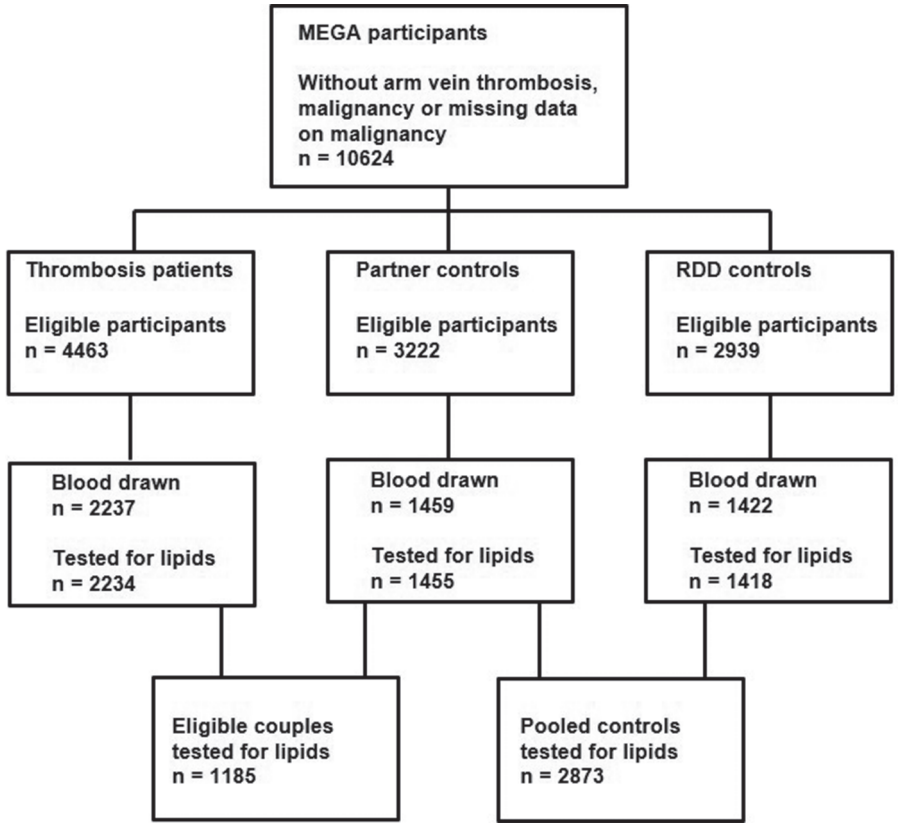

Figure 2. Flow chart of the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study. *RDD* random-digit dialing.

obesity. The index date was defined as the date of diagnosis of venous thrombosis for patients and their partners, and the date of completing the questionnaire for RDD controls. At least 3 months after discontinuation of anticoagulation, or during anticoagulant therapy in patients who continued this therapy for more than 1 year, patients and controls visited the anticoagulation clinic for an interview and blood sampling.

Laboratory measurements

Lipids were measured on stored (-80°C) and previously unthawed fasting serum samples. TC and triglycerides were measured by a colorimetric method (CHOD-PAP for TC and GPO-PAP for triglycerides) on a Modular P analyser (Roche Diagnostics, Mannheim, Germany). HDL-C was measured by a direct method based on the Kyowa Medex reaction principle using polyethylene glycol (PEG)-modified enzymes (Roche

Diagnostics, Mannheim, Germany). Apo A1 and apo B were measured by immunoturbidimetric assays on a Cobas Integra analyzer (Roche Diagnostics, Mannheim, Germany). LDL-C levels were estimated using the Friedewald formula [LDL-C = TC - HDL-C - (triglycerides/2.2) for mmol/L] [25], and when triglycerides exceeded 4.52 mmol/L, LDL-C was not estimated. The natural anticoagulants (antithrombin, protein C, and total protein S), the procoagulant factors (fibrinogen, factors II, VII, VIII, IX, X and XI, and von Willebrand factor), clot lysis time, and CRP levels were determined according to methods previously described [26,27]. Total tissue factor pathway inhibitor (TFPI) activity levels were assessed in citrated plasma by measuring TFPI inhibition of the catalytic tissue factor (TF)-factor VIIa (FVIIa) complex using the Actichrome TFPI activity assay (Sekisui Diagnostics, Stamford, CT, USA); one unit of TFPI activity corresponds to 55 ng/ml plasma TFPI. All laboratory analyses were performed without knowledge of whether the sample was from a patient or a control subject.

Statistical analyses

Demographic and clinical characteristics related to lipid levels in controls

To obtain insight in potential confounding variables, we estimated in the pooled control group (partner and RDD controls) mean differences and their 95% confidence intervals (CIs) in lipid levels by linear regression in relation to age (50-70 years versus 18-50 years [reference]), sex (women versus men [reference]), BMI (overweight/obesity versus normal weight [reference]), self-reported diabetes (yes versus no [reference]), estrogen use at blood sampling (users versus nonusers [reference]), and statin use (users versus nonusers [reference]). All lipids were normally distributed, with the exception of triglycerides (right-skewed distribution), which levels were log-transformed. In our regression models, each lipid was entered as the dependent variable, and the demographic or clinical characteristics (i.e., age, sex, BMI, self-reported diabetes, estrogen use at blood sampling, and statin use) were the independent variables. The resulting regression coefficient (β) for a clinical or demographic characteristic indicated the mean difference in lipid levels between the reference and the other category of that particular characteristic. When applicable, mean differences and their 95% CIs were adjusted for age (continuous) and sex, and further for the other aforementioned characteristics.

Lipid levels and risk of venous thrombosis

Lipid categories were defined according to the values measured in the pooled control group (<10^{th}, 10^{th}-25^{th}, 25^{th}-75^{th}, 75^{th}-90^{th}, and >90^{th} percentile). Age- and sex-adjusted odds ratios and their 95% CIs were calculated as estimates of the relative risk of venous

thrombosis for the different lipid categories in comparison with the reference category (25^{th}-75^{th} percentiles) by unconditional logistic regression. We further adjusted for other potential confounders to assess whether an increased thrombosis risk could be explained by these factors i.e.: estrogen use at blood sampling (dichotomous value), BMI (continuous values), statin use (dichotomous value), and self-reported diabetes (dichotomous value).

Dyslipidemia may be related to lifestyle [2], which may affect venous thrombosis risk [17], and therefore lifestyle may act as another confounder. Such behavior is not easily measured and adjusted for. Partners of patients are likely to resemble the patients in lifestyle, and therefore we performed an additional 1:1 matched analysis by conditional logistic regression which adjusts for associations within matched couples. This method fully takes matching into account, with adjustment for all unmeasured factors for which couples tend to be similar (e.g., socioeconomic class) [28]. In this analysis, all aforementioned potential confounders were additionally adjusted for as covariates. Although using partners as controls results in most controls having the opposite sex as their matched case, one can adjust for sex in a partner-matched case-control study by adding sex to the model [28].

Mediation analyses

In case an association between lipid levels and venous thrombosis is present, a mediation analysis is useful to assess whether this association could be explained by factors related to hemostasis and inflammation. First, we investigated whether lipid levels were associated with changes in hemostatic factor and CRP levels in the general population by evaluating the pooled control group. For this purpose, we used linear regression to estimate mean differences and their 95% CIs in levels of hemostatic factors and CRP for the 10^{th}-25^{th}, 25^{th}-75^{th}, 75^{th}-90^{th}, and $>90^{th}$ percentile categories of lipid levels in comparison with the reference category ($<10^{th}$ percentile). Because CRP levels showed a distribution skewed to the right, a log-transformation was applied for this variable. In our regression models, CRP (log-transformed) or each of the hemostatic factors studied was entered as the dependent variable, and the aforementioned lipid categories were the independent variables. The resulting β coefficient for a lipid category indicated the mean difference in levels of hemostatic factors or CRP between that particular category and the reference. Mean differences and their 95% CIs were adjusted for age, sex, estrogen use at blood sampling, BMI, statin use, and self-reported diabetes. We further used linear regression to estimate the increase or decrease in levels of hemostatic factors or CRP (log-transformed) for every 1 unit increase in lipid levels after adjustment for the same potential confounders. Controls (n =27) who used vitamin K antagonists at the time of blood draw were excluded from the analyses of the vitamin K-dependent factors.

Second, we repeated the earlier logistic regression analyses, at this time including factors associated with lipid levels. Hemostatic factors and CRP (log-transformed) were introduced in the logistic regression model as continuous variables. Patients (n=271) and controls (n=27) using vitamin K antagonists at the time of blood draw were excluded if vitamin K-dependent factors were added to the model.

All statistical analyses were performed with SPSS for Windows, release 20.0 (SPSS Inc, Chicago, IL).

RESULTS

Clinical characteristics

Assessment of lipids was successful for over 99% of the eligible participants, with a total of 2234 patients, 1455 partner and 1418 RDD controls (Figure 2). Of the 1455 partners, 1185 were matched with patients. Table 1 shows the baseline characteristics of the participants. There were no substantial age and sex differences between patients and controls. BMI was higher in patients than in RDD controls and it was virtually the same as in partners. Patients used hormones more often at the index date than controls, and controls were more likely to use hormones at blood sampling, as most patients discontinued hormone use after venous thrombosis. The percentage of self-reported diabetes was virtually the same among participants, and controls used statins more often than patients. The median time between the thrombotic event and blood collection was 10.0 months (interquartile range: 8.3-12.4 months). Supplementary Table 1 (Online Resource) shows no substantial differences in the baseline characteristics of all participants compared with those who provided blood samples, indicating that the tested individuals were representative of the whole MEGA group.

Demographic and clinical characteristics related to lipid levels in controls

Variation in lipid levels according to demographic and clinical characteristics in controls is detailed in Supplementary Table 2 (Online Resource). Lipid levels increased with age, and men (as compared with women) had higher levels of LDL-C, triglycerides, and apo B and lower levels of HDL-C and apo A1. Levels of HDL-C and apo A1 were lower, whereas levels of the other lipids were higher in overweight/obese controls than in those with normal weight. TC, LDL-C, and apo B were, as expected, reduced among statin users. Estrogen users had higher levels of triglycerides, apo B, and apo A1 as compared with nonusers. Except for triglycerides, self-reported diabetes was associated with a reduction in lipid levels.

Table 1. Baseline characteristics

	Patients n = 2234		**Partners n = 1455**		**RDD n = 1418**		**Partners + RDD n = 2873**	
Age, years	49	(26-67)	51	(29-66)	48	(24-67)	50	(27-67)
Women, n (%)	1217	(55)	746	(51)	763	(54)	1509	(53)
BMI (Kg/m^2)	26	(20-35)	26	(20-33)	25	(20-32)	25	(20-33)
Estrogen use at index date, n (% in women)	726	(61)	179	(25)	227	(30)	406	(28)
Estrogen use at blood sampling, n (% in women)	205	(18)	159	(23)	222	(30)	381	(26)
Statin use, n (%)	72	(3)	75	(5)	108	(8)	183	(6)
Self-reported diabetes, n (%)	72	(3)	46	(3)	41	(3)	87	(3)
Venous thrombosis								
DVT, n (%)	1307	(59)	NA		NA		NA	
PE ± DVT, n (%)	927	(41)	NA		NA		NA	

Continuous variables are shown as median (5th percentile - 95th percentile) and categorical variables as number (%).

Data were missing for some participants in some subgroups.

RDD random-digit dialing, *BMI* body mass index, *DVT* deep vein thrombosis, *PE* pulmonary embolism, *NA* not applicable.

Lipid levels and risk of venous thrombosis

Table 2 lists the risk of venous thrombosis for percentile categories of lipid levels. When partners and RDD controls were pooled as the control group, TC, LDL-C, and triglycerides levels were not associated with venous thrombosis in the age- and sex-adjusted model and after full adjustment. In contrast, decreasing apo B levels were associated with an increasing venous thrombosis risk in the pooled control analyses. In the age- and sex-adjusted model, the lowest percentile category of apo B as compared with the reference category resulted in a 1.35-fold (95% CI 1.12- 1.62) increased risk of venous thrombosis (Table 2), and the association was strengthened with further adjustment for BMI (odds ratio = 1.56, 95% CI 1.28-1.89). Addition of the other potential confounders to the model did not substantially change risk estimates, which increased dose-dependently with the reduction of apo B levels across percentile categories (Table 2).

In analyses using both control groups, decreasing HDL-C and apo A1 levels were associated with an increasing risk of venous thrombosis in a dose-dependent manner (Table 2). Compared with the reference categories, the lowest percentile categories of HDL-C and apo A1 yielded odds ratio for venous thrombosis of 1.35 (95% CI 1.11-1.62) and 1.50 (95% CI 1.25-1.79), respectively. With adjustment for BMI, the risk conferred by apo A1 was attenuated (odds ratio = 1.33, 95% CI 1.10-1.60), whereas the risk conferred by HDL-C virtually disappeared (odds ratio = 1.10, 95% CI 0.91-

Table 2. Risk of venous thrombosis by percentiles of lipid levels

	Patients (n=2234)		Controls (n=2873)		Pooled control analyses				Partner control analyses			
					OR (95%CI)[a]		OR (95%CI)[b]		OR (95%CI)[a]		OR (95%CI)[b]	
TC (mmol L^{-1})												
< 10th (<4.28)	217	(10)	286	(10)	0.94	(0.78-1.15)	1.04	(0.84-1.29)	1.09	(0.80-1.49)	1.21	(0.87-1.69)
10th - 25th (4.28-4.84)	346	(15)	428	(15)	1.02	(0.87-1.21)	1.06	(0.89-1.26)	0.87	(0.69-1.11)	0.98	(0.75-1.27)
25th - 75th (4.84-6.30)	1107	(50)	1431	(50)	1	(reference)	1	(reference)	1	(reference)	1	(reference)
75th - 90th (6.30-7.04)	332	(15)	441	(15)	0.98	(0.83-1.16)	0.96	(0.81-1.14)	0.74	(0.59-0.94)	0.78	(0.61-1.01)
> 90th (>7.04)	232	(10)	287	(10)	1.07	(0.88-1.29)	1.00	(0.82-1.22)	0.80	(0.61-1.06)	0.82	(0.60-1.11)
LDL-C (mmol L^{-1})												
< 10th (<2.38)	213	(10)	282	(10)	0.92	(0.76-1.12)	1.09	(0.88-1.35)	1.16	(0.85-1.59)	1.42	(1.01-1.99)
10th - 25th (2.38-2.87)	304	(14)	425	(15)	0.88	(0.75-1.04)	0.97	(0.81-1.15)	0.98	(0.75-1.26)	1.05	(0.79-1.39)
25th - 75th (2.87-4.17)	1118	(51)	1414	(50)	1	(reference)	1	(reference)	1	(reference)	1	(reference)
75th - 90th (4.17-4.85)	342	(15)	425	(15)	1.03	(0.87-1.21)	1.02	(0.86-1.21)	0.92	(0.73-1.15)	0.88	(0.68-1.13)
> 90th (>4.85)	228	(10)	282	(10)	1.05	(0.86-1.27)	0.98	(0.80-1.20)	0.93	(0.71-1.22)	0.96	(0.71-1.29)
Triglycerides (mmol L^{-1})												
< 10th (<0.79)	224	(10)	273	(10)	1.02	(0.84-1.25)	1.14	(0.92-1.40)	1.26	(0.92-1.72)	1.39	(0.99-1.95)
10th - 25th (0.79-1.00)	309	(14)	441	(15)	0.89	(0.75-1.05)	0.95	(0.80-1.14)	0.94	(0.73-1.21)	1.01	(0.77-1.32)
25th - 75th (1.00-1.88)	1104	(49)	1440	(50)	1	(reference)	1	(reference)	1	(reference)	1	(reference)
75th - 90th (1.88-2.58)	355	(16)	432	(15)	1.09	(0.93-1.29)	1.05	(0.88-1.24)	1.01	(0.80-1.27)	0.98	(0.76-1.26)
> 90th (>2.58)	242	(11)	287	(10)	1.13	(0.93-1.36)	0.96	(0.79-1.18)	0.95	(0.72-1.24)	0.89	(0.66-1.20)

Table 2. (continued)

	Patients (n=2234)		Controls (n=2873)		Pooled control analyses				Partner control analyses			
					OR (95%CI)[a]		OR (95%CI)[b]		OR (95%CI)[a]		OR (95%CI)[b]	
Apo B (g L^{-1})												
< 10th (<0.68)	292	(13)	285	(10)	1.35	(1.12-1.62)	**1.54**	**(1.26-1.88)**	1.30	(0.99-1.72)	**1.49**	**(1.10-2.00)**
10th - 25th (0.68-0.80)	356	(16)	431	(15)	1.10	(0.93-1.29)	**1.20**	**(1.01-1.42)**	1.01	(0.79-1.28)	**1.19**	**(0.92-1.54)**
25th - 75th (0.80-1.15)	1071	(48)	1435	(50)	1	(reference)	**1**	**(reference)**	1	(reference)	**1**	**(reference)**
75th - 90th (1.15-1.33)	313	(14)	425	(15)	1.00	(0.85-1.18)	**0.91**	**(0.76-1.08)**	0.95	(0.74-1.21)	**1.06**	**(0.81-1.38)**
> 90th (>1.33)	202	(9)	297	(10)	0.92	(0.75-1.12)	**0.84**	**(0.68-1.03)**	0.98	(0.73-1.33)	**1.01**	**(0.73-1.40)**
HDL-C (mmol L^{-1})												
< 10th (<0.90)	272	(12)	289	(10)	1.35	(1.11-1.62)	1.12	(0.91-1.37)	1.04	(0.79-1.38)	0.90	(0.66-1.23)
10th - 25th (0.90-1.07)	387	(17)	434	(15)	1.26	(1.07-1.48)	1.17	(0.99-1.39)	1.08	(0.85-1.36)	1.04	(0.81-1.34)
25th - 75th (1.07-1.56)	1068	(48)	1449	(50)	1	(reference)	1	(reference)	1	(reference)	1	(reference)
75th - 90th (1.56-1.86)	330	(15)	421	(15)	1.03	(0.87-1.22)	1.12	(0.94-1.33)	1.12	(0.87-1.44)	1.14	(0.86-1.49)
> 90th (>1.86)	177	(8)	280	(10)	0.82	(0.67-1.01)	0.97	(0.78-1.21)	0.79	(0.58-1.09)	0.80	(0.57-1.13)
Apo A1 (g L^{-1})												
< 10th (<1.09)	322	(14)	295	(10)	1.50	(1.25-1.79)	**1.36**	**(1.12-1.64)**	1.26	(0.97-1.65)	**1.19**	**(0.89-1.60)**
10th - 25th (1.09-1.22)	347	(16)	429	(15)	1.09	(0.93-1.29)	**1.02**	**(0.86-1.21)**	0.98	(0.77-1.24)	**0.91**	**(0.70-1.18)**
25th - 75th (1.22-1.59)	1107	(50)	1444	(50)	1	(reference)	**1**	**(reference)**	1	(reference)	**1**	**(reference)**
75th - 90th (1.59-1.81)	296	(13)	432	(15)	0.86	(0.73-1.02)	**0.96**	**(0.80-1.15)**	0.84	(0.66-1.08)	**0.91**	**(0.69-1.19)**
> 90th (>1.81)	162	(7)	273	(10)	0.73	(0.59-0.90)	**0.88**	**(0.70-1.10)**	0.66	(0.47-0.93)	**0.67**	**(0.47-0.97)**

Data were missing for some participants in some subgroups.

OR odds ratio, *CI* confidence interval, *TC* total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *Apo B* apolipoprotein B, *HDL-C* high-density lipoprotein cholesterol, *Apo A1* apolipoprotein A1.

[a]Adjusted for age, sex and partnership (where applicable).

[b]Adjusted for age, sex, body mass index, estrogen use at blood sampling, statin use, self-reported diabetes, and partnership (where applicable).

OR[b] (full adjustment) for apo B and apo A1 is in bold.

1.35). After full adjustment, both risk estimates did not substantially change, and the dose-response relation remained between apo A1 levels and venous thrombosis risk, as depicted in Table 2.

When the analysis was restricted to partners as the control group, there was again no consistent association between venous thrombosis and levels of TC, LDL-C, triglycerides and HDL-C across percentile categories (Table 2). Results for apo B and apo A1 were similar to those obtained with the pooled control group, i.e., risk estimates for venous thrombosis increased with decreasing levels of apo B or apo A1.

As risks of venous thrombosis were apparently not related with TC, LDL-C, triglycerides and HDL-C, these lipids were not further considered in the subsequent analyses.

Mediation analyses

In comparison with the reference category, an increment in apo B levels across percentile categories was associated with a consistent increase in levels of natural anticoagulants (protein C, protein S, antithrombin, and TFPI), procoagulant factors (fibrinogen, and factors II, VII, IX, X, and XI), clot lysis time, and CRP (Table 3). With the exception of fibrinogen, changes in hemostatic factor levels were in the same direction for apo A1, i.e., as apo A1 increased, levels of protein C, and factors II, VII, IX, X, and XI increased as well (Table 4). Among the procoagulant factors, only factor VIII and von Willebrand factor were not associated with apo B and apo A1. The behavior of the aforementioned hemostatic factor and CRP levels was the same when apo B and apo A1 were introduced continuously in the model.

When CRP and the hemostatic factors associated with apo B or apo A1 were added to the logistic regression model (Table 5), risk estimates changed marginally for either apolipoprotein as compared with the analyses adjusted for all potential confounders, regardless the type of control group used. When the potential mediators were entered one by one in the model, results were similar to those described in Table 5 for both apolipoproteins (data not shown).

Table 3. Effect of percentiles of apolipoprotein B on the levels of hemostatic factors and c-reactive protein in control subjects

	Mean levels reference	Adjusted[a] mean difference (95% CI) compared with the reference category								Apo B continuous scale	
	<10th Percentile (Apo B, <0.68g L^{-1}) (n=285)	10th-25th Percentile (Apo B, 0.68-0.80g L^{-1}) (n=431)		25th-75th Percentile (Apo B, 0.80-1.15g L^{-1}) (n=1435)		75th-90th Percentile (Apo B, 1.15-1.33g L^{-1}) (n=425)		>90th Percentile (Apo B, >1.33g L^{-1}) (n=297)		Apo B per 1 g L^{-1} increase (n=2873)	
Hemostatic factors											
Anticoagulant factors											
Protein C (IU/dL)[b]	105	4	(1 to 7)	12	(9 to 14)	19	(16 to 23)	25	(21 to 30)	27	(24 to 31)
Protein S antigen (IU/dL)[b]	91	2	(-1 to 4)	8	(6 to 11)	13	(10 to 16)	16	(13 to 20)	21	(18 to 24)
Antithrombin (IU/dL)	103	3	(1 to 4)	5	(3 to 6)	7	(5 to 9)	7	(5 to 9)	8	(6 to 9)
TFPI activity (U/dL)	144	9	(4 to 15)	28	(22 to 34)	42	(34 to 49)	42	(34 to 50)	59	(53 to 66)
Procoagulant factors											
Fibrinogen (g/L)	3.1	0	(-0.1 to 0.1)	0.1	(0 to 0.2)	0.2	(0.1 to 0.3)	0.2	(0.1 to 0.4)	0.4	(0.3 to 0.5)
Factor II (IU/dL)[b]	103	3	(1 to 5)	9	(7 to 11)	15	(11 to 18)	18	(15 to 21)	22	(20 to 24)
Factor VII (IU/dL)[b]	98	4	(0 to 7)	11	(8 to 14)	16	(12 to 19)	23	(18 to 27)	24	(21 to 28)
Factor VIII (IU/dL)	110	-5	(-10 to 1)	-3	(-8 to 2)	-4	(-11 to 3)	2	(-6 to 10)	3	(-3 to 8)
VWF (IU/dL)	108	-8	(-15 to 0)	-6	(-12 to 0)	-7	(-17 to 3)	1	(-10 to 12)	4	(-4 to 10)
Factor IX antigen (IU/dL)[b]	95	2	(0 to 4)	7	(5 to 10)	10	(7 to 13)	15	(12 to 19)	17	(15 to 20)
Factor X (IU/dL)[b]	108	3	(1 to 6)	10	(8 to 12)	15	(12 to 18)	21	(17 to 24)	23	(21 to 26)
Factor XI (IU/dL)	93	2	(-1 to 5)	7	(5 to 10)	12	(9 to 16)	11	(7 to 15)	15	(12 to 17)
Fibrinolytic factor											
Clot lysis time (min)[b]	61	1	(-1 to 3)	6	(4 to 9)	10	(7 to 14)	14	(10 to 17)	17	(14 to 19)
Inflammatory marker											
CRP (mg/L)[c]	0.2	-0.1	(-0.2 to 0.1)	0.1	(0 to 0.2)	0.2	(0 to 0.3)	0.3	(0.1 to 0.5)	0.4	(0.3 to 0.6)

Data were missing for some participants in some subgroups. *Apo B* apolipoprotein B, *CI* confidence interval, *TFPI* tissue factor pathway inhibitor, *VWF* von Willebrand factor, *CRP* C-reactive protein. [a] Adjusted for age, sex, body mass index, estrogen use at blood sampling, statin use, and self-reported diabetes. [b] Vitamin K antagonists users at the time of blood sampling were excluded from the analyses. [c] Log-transformed.

Table 4. Effect of percentiles of apolipoprotein A1 on the levels of hemostatic factors and c-reactive protein in control subjects

	Mean levels reference	Adjusted[a] mean difference (95% CI) compared with the reference category								ApoA1 continuous scale	
	<10th Percentile (Apo A1, <1.09g L^{-1}) (n=295)	10th-25th Percentile (Apo A1, 1.09-1.22g L^{-1}) (n=429)		25th-75th Percentile (Apo A1, 1.22-1.59g L^{-1}) (n=1444)		75th-90th Percentile (Apo A1, 1.59-1.81g L^{-1}) (n=432)		>90th Percentile (Apo A1, >1.81g L^{-1}) (n=273)		Apo A1 per 1 g L^{-1} increase (n=2873)	
Hemostatic factors											
Anticoagulant factors											
Protein C (IU/dL)[b]	111	4	(1 to 7)	6	(3 to 8)	12	(8 to 16)	15	(10 to 20)	15	(12 to 18)
Protein S antigen (IU/dL)[b]	101	2	(-1 to 5)	1	(-2 to 3)	1	(-2 to 5)	0	(-4 to 4)	-2	(-5 to 1)
Antithrombin (IU/dL)	105	0	(-2 to 2)	0	(-2 to 1)	0	(-2 to 2)	1	(-2 to 4)	0	(-1 to 2)
TFPI activity (U/dL)	177	-2	(-9 to 5)	-4	(-10 to 2)	0	(-9 to 9)	-11	(-22 to 0)	-3	(-10 to 4)
Procoagulant factors											
Fibrinogen (g/L)	3.5	-0.2	(-0.3 to -0.1)	-0.3	(-0.4 to -0.2)	-0.4	(-0.5 to -0.2)	-0.4	(-0.6 to -0.2)	-0.3	(-0.4 to -0.2)
Factor II (IU/dL)[b]	109	2	(-1 to 5)	0	(-1 to 2)	2	(-1 to 5)	7	(3 to 11)	6	(4 to 8)
Factor VII (IU/dL)[b]	101	6	(2 to 9)	7	(4 to 10)	14	(9 to 18)	21	(15 to 26)	16	(13 to 20)
Factor VIII (IU/dL)	108	-1	(-7 to 4)	-1	(-6 to 4)	6	(-2 to 15)	3	(-6 to 13)	3	(-2 to 9)
VWF (IU/dL)	107	2	(-6 to 10)	-1	(-6 to 5)	3	(-5 to 11)	8	(-2 to 18)	1	(-6 to 7)
Factor IX antigen (IU/dL)[b]	103	1	(-2 to 3)	0	(-2 to 2)	2	(-2 to 5)	7	(3 to 12)	4	(2 to 6)
Factor X (IU/dL)[b]	113	3	(0 to 6)	5	(2 to 7)	8	(5 to 11)	18	(14 to 22)	14	(11 to 17)
Factor XI (IU/dL)	95	2	(-1 to 5)	3	(0 to 5)	4	(0 to 8)	6	(2 to 10)	5	(2 to 8)
Fibrinolytic factor											
Clot lysis time (min)[b]	72	0	(-3 to 3)	-1	(-3 to 2)	-2	(-6 to 2)	-2	(-6 to 2)	-1	(-4 to 1)
Inflammatory marker											
CRP (mg/L)[c]	0.6	-0.2	(-0.3 to 0)	-0.4	(-0.5 to -0.2)	-0.3	(-0.5 to -0.1)	-0.2	(-0.4 to 0.1)	-0.2	(-0.3 to -0.1)

Data were missing for some participants in some subgroups. *Apo A1* apolipoprotein A1, *CI* confidence interval, *TFPI* tissue factor pathway inhibitor, *VWF* von Willebrand factor, CRP *C-reactive protein.* [a] Adjusted for age, sex, body mass index, estrogen use at blood sampling, statin use, and self-reported diabetes. [b] Vitamin K antagonists users at the time of blood sampling were excluded from the analyses. [c] Log-transformed.

Table 5. Risk of venous thrombosis by percentiles of apolipoproteins B and A1 levels adjusted for hemostatic factors and c-reactive protein

	Pooled control analyses						Partner control analyses					
	OR	**(95%CI)[a]**	**OR**	**(95%CI)[ab]**	**OR**	**(95%CI)[abc]**	**OR**	**(95%CI)[a]**	**OR**	**(95%CI)[ab]**	**OR**	**(95%CI)[abc]**
Apo B (g L^{-1})												
< 10th (<0.68)	1.38	(1.14-1.67)	1.58	(1.28-1.93)	**1.86**	**(1.49-2.31)**	1.29	(0.97-1.71)	1.44	(1.06-2.00)	**1.59**	**(1.12-2.25)**
10th - 25th (0.68-0.80)	1.13	(0.95-1.33)	1.22	(1.02-1.45)	**1.36**	**(1.13-1.64)**	1.04	(0.81-1.34)	1.22	(0.93-1.59)	**1.29**	**(0.97-1.72)**
25th - 75th (0.80-1.15)	1	(reference)	1	(reference)	**1**	**(reference)**	1	(reference)	1	(reference)	**1**	**(reference)**
75th - 90th (1.15-1.33)	0.98	(0.83-1.17)	0.88	(0.74-1.06)	**0.81**	**(0.67-0.98)**	0.98	(0.76-1.28)	1.09	(0.82-1.45)	**1.01**	**(0.74-1.37)**
> 90th (>1.33)	0.80	(0.65-0.99)	0.72	(0.58-0.90)	**0.63**	**(0.49-0.80)**	0.84	(0.60-1.17)	0.88	(0.62-1.26)	**0.88**	**(0.59-1.31)**
Apo A 1 (g L^{-1})												
< 10th (<1.09)	1.42	(1.18-1.72)	1.29	(1.05-1.57)	**1.20**	**(0.98-1.48)**	1.24	(0.93-1.65)	1.17	(0.85-1.59)	**1.09**	**(0.78-1.52)**
10th - 25th (1.09-1.22)	1.08	(0.91-1.28)	1.01	(0.84-1.20)	**0.98**	**(0.82-1.18)**	1.03	(0.80-1.33)	0.96	(0.73-1.27)	**0.97**	**(0.73-1.30)**
25th - 75th (1.22-1.59)	1	(reference)	1	(reference)	**1**	**(reference)**	1	(reference)	1	(reference)	**1**	**(reference)**
75th - 90th (1.59-1.81)	0.88	(0.73-1.04)	0.99	(0.83-1.20)	**1.02**	**(0.85-1.24)**	0.83	(0.63-1.08)	0.88	(0.66-1.17)	**0.91**	**(0.67-1.22)**
> 90th (>1.81)	0.71	(0.56-0.88)	0.86	(0.68-1.09)	**0.84**	**(0.66-1.08)**	0.65	(0.45-0.93)	0.65	(0.44-0.96)	**0.67**	**(0.46-1.00)**

Data were missing for some participants in some subgroups.

OR odds ratio, *CI* confidence interval, *Apo B* apolipoprotein B, *apo A1* apolipoprotein A1.

[a]Adjusted for age, sex and partnership (where applicable).

[b]Adjusted for body mass index, estrogen use at blood sampling, statin use, and self-reported diabetes.

[c]Adjusted for potential mediators for apolipoprotein B (protein C, protein S, antithrombin, tissue factor pathway inhibitor, fibrinogen, factors II, VII, IX, X, and XI, clot lysis time, and c-reactive protein) or for apolipoprotein A1 (protein C, fibrinogen, factors II, VII, IX, X, and XI, and c-reactive protein).

OR^{a} and OR^{ab} were slightly different from those depicted in Table 2 because vitamin K antagonist users at the time of blood sampling (n = 271 patients and n = 27 controls) were excluded from the mediation analyses.

OR^{abc} (further adjustment for potential mediators) is in bold.

DISCUSSION

In this large population-based case-control study, levels of the major lipids (i.e., TC, LDL-C, triglycerides, or HDL-C) were not associated with an increased risk of venous thrombosis. In contrast, apo B and apo A1 appeared inversely associated with venous thrombosis, as decreasing levels of both apolipoproteins were associated with an increased risk of venous thrombosis, also after adjustment for potential confounders. Although apo B and to a lesser extent apo A1 were associated with several hemostatic factors and CRP, none of these factors explained the association between these apolipoproteins and venous thrombosis risk.

A previous meta-analysis demonstrated that mean levels of triglycerides were higher and those of HDL-C lower in venous thrombosis patients than in controls [6]. However, the majority of the reports on lipids and venous thrombosis in this meta-analysis were small case-control studies, and controlling for several confounders had not been possible [6]. Our results follow the majority of the longitudinal studies published after the aforementioned meta-analysis [6], that collectively showed little to no evidence of an association between the major lipid levels and risk of venous thrombosis [7,10-12].

In contrast to the major lipids, fewer reports have addressed the relationship between venous thrombosis and levels of apo B and apo A1. In two cohort studies [8,12] neither apolipoprotein was associated with risk of venous thrombosis. In the other reports, an association between these apolipoproteins and venous thrombosis was restricted to certain subgroups. For instance, in a small case-control study composed of men only, low apo A1 levels were associated with an increased risk of venous thrombosis [29]. On the other hand, high apo A1 levels in the *Women's Health Study* were associated with an increased thrombosis risk in hormone users [9]. In a hospital-based case-control study, high apo B levels appeared to increase the risk of venous thrombosis mainly in men [13]. Differences related to the study design, the source and selection criteria of the study population, the sample size, and the adjustment for potential confounders might have contributed to the contradictory results among these studies [8,9,12,13,29].

After excluding a major role for the most important confounders in the association between venous thrombosis and levels of apo B and apo A1, we investigated whether this association might be explained by potential mediators. Although apo B and to a lesser extent apo A1 levels were associated with levels of several hemostatic factors and CRP, their inclusion in the logistic regression model only marginally affected risk estimates conferred by both apolipoproteins, thereby excluding a mediating role for these factors. Nevertheless, the strong association between levels of apolipoproteins and hemostatic factors/CRP deserves some further attention. We can hypothesize that this may reflect a common mechanism, that influences the synthesis of these proteins

in hepatocytes [30-32]. In line with this hypothesis was the finding that factor VIII and von Willebrand factor, both largely expressed by endothelial cells [31,33], were not associated with apo B or apo A1 levels. TFPI fits this hypothesis with respect to apo A1 (no relation found), as it is also primarily synthesized by endothelial cells [34]. The association between total TFPI activity and apo B levels that was nevertheless found is consistent with the fact that the majority of the TFPI in plasma is bound to apo B-containing lipoproteins, mainly LDL [34].

Here we observed a protective role for apo B against venous thrombosis. Conversely, there is strong evidence that elevated apo B and LDL-C are associated with increased risk for arterial disease [35,36]. Still, one should consider that even though arterial and venous thrombosis share some risk factors [1], their pathophysiology is different. An arterial thrombus is typically formed after rupture of an atherosclerotic plaque, whereas venous thrombi assemble on the surface of a largely intact vessel wall [37]. Based on this, it would be reasonable to consider that apo B may play different roles in the mechanisms that are at the basis of venous versus arterial thrombosis. Indeed, our results on the inverse association of apo B levels with risk of venous thrombosis are consistent with experimental studies demonstrating that apo B is capable of inhibiting coagulation [38,39]. *In vitro*, purified human apo B was shown to inhibit TF-initiated coagulation either alone or reconstituted into LDL-like particles in a thus far undefined TFPI-independent manner [39]. Alternatively, apo B could indirectly exert an anticoagulant function via LDL-bound TFPI. The latter was shown to inhibit the coagulation protease factor Xa activity more potently as compared with the TF-FVIIa inhibition [40]. In the present study, we assessed total plasma TFPI activity via inhibition of the TF-FVIIa complex, which might explain why TFPI did not mediate, at least in part, the association between apo B and venous thrombosis risk.

It might be argued that our findings on apo B and venous thrombosis are not consistent with the putative protection conferred by statins against venous thrombosis [3-5], since these drugs decrease apo B levels [41,42]. It is noteworthy that statins may have antithrombotic effects that are unrelated to their lipid-lowering activity, such as downregulation of TF [43]. It is currently unknown, however, to what extent the antithrombotic potential of statins would influence the thrombosis risk or the anticoagulant properties of apo B demonstrated *in vitro* [38,39].

Unlike apo B, risk estimates for venous thrombosis associated with apo A1 pointed in the same direction to that reported in observational studies on arterial disease [36]. Importantly, our results are consistent with experimental data on the role of this apolipoprotein in protecting mice against venous thrombosis [44]. It has been demonstrated that flow restriction-induced venous thrombosis was more common in apo $A1^{-/-}$ mice as compared with wild-type mice, and intravenous infusion of human

apo A1 prevented venous thrombosis in wild-type mice but not in mice lacking scavenger receptor B type I or endothelial nitric oxide synthase [44]. The interaction between apo A1 within HDL-C and scavenger receptor B type I promotes not only the reverse cholesterol transport but also has been shown to stimulate the activation of endothelial nitric oxide synthase [45], which results in the production of nitric oxide. An impairment in nitric oxide bioavailability represents a central feature of endothelial cell activation and dysfunction [46]. As endothelial activation could contribute to venous thrombus formation [37], according to the the murine model study [44] one of the possible mechanisms by which apo A1 may protect against venous thrombosis is the upregulation of nitric oxide production and as a result the maintenance of endothelial integrity. Additionally, apo A1 has been suggested to have anticoagulant properties *in vitro*, as apo A1 was observed to play a critical role in the ability of HDL to enhance the anticoagulant potential of the protein C pathway [21,47].

The strengths of this study include the large patient sample and the detailed assessment of the relationship between lipids and venous thrombosis, considering not only confounding by several common risk factors, but also mediation via hemostatic factors. Estimation of venous thrombosis risk using only partners as controls enabled us to adjust for further confounding by socio-economic factors [28]. Importantly, variation in lipid levels according to demographic and clinical characteristics in controls (age, sex, BMI, and estrogen- and statin-use) agreed with previous findings [2,14,15,18,19]. Furthermore, our results contributed to clarify the protective role of apo A1 against venous thrombosis, confirming prior findings from a case-control study [29] and a murine model of venous thrombosis [44]. Our study is the first, to our knowledge, to show that apo B levels are also inversely associated with risk of venous thrombosis, a finding supported by the anticoagulant properties of this apolipoprotein observed *in vitro* [38,39].

Since this is an etiologic study, alternative explanations for our results should be addressed and considered as potential limitations. First, we cannot rule out that chance has played a role in our results. Still, chance seems an unlikely explanation owing to the dose-response association between the risk of venous thrombosis and levels of apo B and apo A1 in most of our models. Second, one could argue that sampling of the blood after the event of venous thrombosis might have resulted in reverse causation, i.e., the venous thrombotic event has led to changes in lipid levels. Indeed, after venous thrombosis, patients may have modified certain lifestyle factors, which could have affected their lipid profile. Randomized clinical trials published during the nineties showed that changes in diet affected lipid levels in individuals with dyslipidemia [48,49]. However, the reduction in the levels of TC, LDL-C and apo B produced by a low-fat diet taken from 9 weeks to 1 year in these trials was small or

virtually absent without concomitant statin use or aerobic-exercise program [48,49]. It is quite unlikely that venous thrombosis patients would systematically adhere to a low-fat diet in addition to statin use and/or exercise after the thrombotic event, especially considering that such recommendation is not routinely given, in contrast to arterial disease. Another mechanism for reverse causation would be the effect of acute phase reactions brought about by the thrombotic event on lipid levels [50,51]. During acute phase reactions in humans, triglyceride levels typically increase and HDL-C and apo A1 levels decrease, whereas TC, LDl-C and apo B levels can either decrease or do not change [50,51]. Nevertheless, this is an unlikely mechanism for reverse causation, since blood was collected with a median of 10 months after the thrombotic event, by which time the effects of the acute-phase reaction will have worn off [52,53]. Third, low cholesterol levels have been reported in some chronic illnesses, such as malignancy, rheumatic disorders, hyperthyroidism and tuberculosis [54]. Hence, these illnesses could have been confounding factors but subjects with malignancy were excluded from the current analyses. Furthermore, changes in lipid levels in our study population owing to reverse causation or chronic illnesses would be expected to affect not only the direction of the point estimates related to the apolipoproteins but also to the other lipids, such as TC, which also makes it unlikely that our findings can be due to either of these explanations. Fourth, even though it is reasonable to assume that patients and their partners likely have a similar lifestyle, lipid levels could be influenced by factors that couples may not share, such as physical activity or diet. Thereby, we cannot rule out the possibility of residual lifestyle-related confounders. However, to reduce this possibility, in the conditional regression analyses we also included in our models BMI, statin use, and diabetes as potential sources of confounding related to lifestyle. Finally, LDL-C was not assessed directly but estimatedby the Friedewald formula [25]. From an analytical perspective, estimation of LDL-C was the sum of the inaccuracies and imprecision of three measurements [TC, HDL-C and triglyceride], whereas apo B was measured by a direct and standardized method [23,55].

In conclusion, in this large, unselected population we showed no association between levels of TC, LDL-C, HDL-C and triglycerides and risk of venous thrombosis. Decreasing levels of apolipoproteins B and A1 were associated with increased risk of venous thrombosis, which we could not explain through several proposed mechanisms, such as confounding or other non-causal explanations, or through mediation via inflammation or changes in the hemostatic factors. Our results may form the basis for further studies to confirm these findings, as well as to assess the physiological relevance of the anticoagulant properties of apo B and apo A1, and to determine the mechanism, whether or not causal, underlying the link between these apolipoproteins and venous thrombosis.

REFERENCES

1. Lijfering WM, Flinterman LE, Vandenbroucke JP, et al. Relationship between venous and arterial thrombosis: a review of the literature from a causal perspective. Semin Thromb Hemost. 2011;37(8):885-896.
2. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation. 2002;106(25):3143-3421.
3. Glynn RJ, Danielson E, Fonseca FA, et al. A randomized trial of rosuvastatin in the prevention of venous thromboembolism. N Engl J Med. 2009;360(18):1851-1861.
4. Ramcharan AS, Van Stralen KJ, Snoep JD, et al. HMG-CoA reductase inhibitors, other lipid-lowering medication, antiplatelet therapy, and the risk of venous thrombosis. J Thromb Haemost. 2009;7(4):514-520.
5. Rahimi K, Bhala N, Kamphuisen P, et al. Effect of statins on venous thromboembolic events: a meta-analysis of published and unpublished evidence from randomised controlled trials. PLoS Med. 2012;9(9):e1001310.
6. Ageno W, Becattini C, Brighton T, et al. Cardiovascular risk factors and venous thromboembolism: a meta-analysis. Circulation. 2008;117(1):93-102.
7. Braekkan SK, Mathiesen EB, Njølstad I, et al. Family history of myocardial infarction is an independent risk factor for venous thromboembolism: the Tromsø study. J Thromb Haemost. 2008;6(11):1851-1857.
8. Chamberlain AM, Folsom AR, Heckbert SR, et al. High-density lipoprotein cholesterol and venous thromboembolism in the Longitudinal Investigation of Thromboembolism Etiology (LITE). Blood. 2008;112(7):2675-2680.
9. Everett BM, Glynn RJ, Buring JE, et al. Lipid biomarkers, hormone therapy and the risk of venous thromboembolism in women. J Thromb Haemost. 2009;7(4):588-596.
10. Quist-Paulsen P, Naess IA, Cannegieter SC, et al. Arterial cardiovascular risk factors and venous thrombosis: results from a population-based, prospective study (the HUNT 2). Haematologica. 2010;95(1):119-125.
11. Holst AG, Jensen G, Prescott E. Risk factors for venous thromboembolism: results from the Copenhagen City Heart Study. Circulation. 2010;121(17):1896-1903.
12. van Schouwenburg IM, Mahmoodi BK, Gansevoort RT, et al. Lipid levels do not influence the risk of venous thromboembolism. Results of a population-based cohort study. Thromb Haemost. 2012;108(5):923-929.
13. Delluc A, Malécot JM, Kerspern H, et al. Lipid parameters, lipid lowering drugs and the risk of venous thromboembolism. Atherosclerosis. 2012;220(1):184-188.
14. Johnson CL, Rifkind BM, Sempos CT, et al. Declining serum total cholesterol levels among US adults. The National Health and Nutrition Examination Surveys. JAMA. 1993;269(23):3002-3008.

15. Bachorik PS, Lovejoy KL, Carroll MD, et al. Apolipoprotein B and AI distributions in the United States, 1988-1991: results of the National Health and Nutrition Examination Survey III (NHANES III). Clin Chem. 1997;43(12):2364-2378.
16. Rosendaal FR. Venous thrombosis: the role of genes, environment, and behavior. Hematology Am Soc Hematol Educ Program. 2005:1-12.
17. Zöller B, Li X, Sundquist J, Sundquist K. Socioeconomic and occupational risk factors for venous thromboembolism in Sweden: a nationwide epidemiological study. Thromb Res. 2012;129(5):577-582.
18. Gardner CD, Tribble DL, Young DR, et al. Population frequency distributions of HDL, HDL(2), and HDL(3) cholesterol and apolipoproteins A-I and B in healthy men and women and associations with age, gender, hormonal status, and sex hormone use: the Stanford Five City Project. Prev Med. 2000;31(4):335-345.
19. Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. BMJ. 2003;326(7404):1423.
20. Petrauskiene V, Falk M, Waernbaum I, et al. The risk of venous thromboembolism is markedly elevated in patients with diabetes. Diabetologia. 2005;48(5):1017-1021.
21. Griffin JH, Fernández JA, Deguchi H. Plasma lipoproteins, hemostasis and thrombosis. Thromb Haemost. 2001;86(1):386-394.
22. Wadham C, Albanese N, Roberts J, et al. High-density lipoproteins neutralize C-reactive protein proinflammatory activity. Circulation. 2004;109(17):2116-2122.
23. Marcovina S, Packard CJ. Measurement and meaning of apolipoprotein AI and apolipoprotein B plasma levels. J Intern Med. 2006;259(5):437-446.
24. van Stralen KJ, Rosendaal FR, Doggen CJ. Minor injuries as a risk factor for venous thrombosis. Arch Intern Med. 2008;168(1):21-26.
25. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972 ;18(6):499-502.
26. Meltzer ME, Lisman T, de Groot PG, et al. Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. Blood. 2010;116(1):113-121.
27. Rezende SM, Lijfering WM, Rosendaal FR, et al. Hematologic variables and venous thrombosis: red cell distribution width and blood monocyte count are associated with an increased risk. Haematologica. 2014;99(1):194-200.
28. Cannegieter SC, Doggen CJ, van Houwelingen HC, et al. Travel-related venous thrombosis: results from a large population-based case control study (MEGA study). PLoS Med. 2006;3(8):e307.
29. Deguchi H, Pecheniuk NM, Elias DJ, et al. High-density lipoprotein deficiency and dyslipoproteinemia associated with venous thrombosis in men. Circulation. 2005;112(6):893-899.

30. Breslow JL. Human apolipoprotein molecular biology and genetic variation. Annu Rev Biochem. 1985;54:699-727.

31. Brummel-Ziedins K, Orfeo T, Jenny NS, et al. Blood coagulation and fibrinolysis. In: Greer JP, Foerster J, Rodgers GM, Paraskevas F, Glader B, Arber DA, Means RT, eds. Wintrobe's Clinical Hematology. Philadelphia: Lippincott Williams & Wilkins; 2009: 528-619.

32. Black S, Kushner I, Samols D. C-reactive Protein. J Biol Chem. 2004;279(47):48487-4890.

33. Shahani T, Covens K, Lavend'homme R, et al. Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. J Thromb Haemost. 2014;12(1):36-42.

34. Crawley JT, Lane DA. The haemostatic role of tissue factor pathway inhibitor. Arterioscler Thromb Vasc Biol. 2008;28(2):233-242.

35. Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet. 2012;380(9841):572-580.

36. Di Angelantonio E, Sarwar N, Perry P, et al. Major lipids, apolipoproteins, and risk of vascular disease. JAMA. 2009;302(18):1993-2000.

37. Mackman N. New insights into the mechanisms of venous thrombosis. J Clin Invest. 2012;122(7):2331-2336.

38. Ettelaie C, Howell RM. The inhibition of thromboplastin by apolipoprotein-B and the effect of various associated lipids. Thromb Res. 1992;68(2):175-184.

39. Ettelaie C, Wilbourn BR, Adam JM, et al. Comparison of the inhibitory effects of ApoB100 and tissue factor pathway inhibitor on tissue factor and the influence of lipoprotein oxidation. Arterioscler Thromb Vasc Biol. 1999;19(7):1784-1790.

40. Augustsson C, Hilden I, Petersen LC. Inhibitory effects of LDL-associated tissue factor pathway inhibitor. Thromb Res. 2014;134(1):132-137.

41. Colhoun HM, Betteridge DJ, Durrington PN, et al. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. Lancet. 2004;364(9435):685-696.

42. Mora S, Caulfield MP, Wohlgemuth J, et al. Atherogenic Lipoprotein Subfractions Determined by Ion Mobility and First Cardiovascular Events After Random Allocation to High-Intensity Statin or Placebo: The Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) Trial. Circulation. 2015; 132(23):2220-2229.

43. Violi F, Calvieri C, Ferro D, et al. Statins as antithrombotic drugs. Circulation. 2013;127(2):251-257.

44. Brill A, Yesilaltay A, De Meyer SF, et al. Extrahepatic high-density lipoprotein receptor SR-BI and apoA-I protect against deep vein thrombosis in mice. Arterioscler Thromb Vasc Biol. 2012;32(8):1841-1848.

45. Mineo C, Deguchi H, Griffin JH, et al. Endothelial and antithrombotic actions of HDL. Circ Res. 2006;98(11):1352-1364.

46. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest. 1997;100(9):2153-2157.

47. Fernandez JA, Deguchi H, Banka CL, et al. Re-evaluation of the anticoagulant properties of high-density lipoprotein-brief report. Arterioscler Thromb Vasc Biol. 2015;35(3):570-572.

48. Hunninghake DB, Stein EA, Dujovne CA, et al. The efficacy of intensive dietary therapy alone or combined with lovastatin in outpatients with hypercholesterolemia. N Engl J Med. 1993; 328(17):1213-1219.

49. Stefanick ML, Mackey S, Sheehan M, et al. Effects of diet and exercise in men and postmenopausal women with low levels of HDL cholesterol and high levels of LDL cholesterol. N Engl J Med. 1998; 339(1):12-20.

50. Khovidhunkit W, Kim MS, Memon RA, et al. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res. 2004;45(7):1169-1196.

51. Esteve E, Ricart W, Fernández-Real JM. Dyslipidemia and inflammation: an evolutionary conserved mechanism. Clin Nutr. 2005;24(1):16-31.

52. O'Donnell J, Mumford AD, Manning RA, et al. Elevation of FVIII: C in venous thromboembolism is persistent and independent of the acute phase response. Thromb Haemost. 2000;83(1):10-13.

53. Tichelaar V, Mulder A, Kluin-Nelemans H, et al. The acute phase reaction explains only a part of initially elevated factor VIII:C levels: a prospective cohort study in patients with venous thrombosis. Thromb Res. 2012;129(2):183-186.

54. Ettinger WH Jr, Harris T. Causes of hypocholesterolemia. Coron Artery Dis. 1993;4(10):854-859.

55. Contois JH, Warnick GR, Sniderman AD. Reliability of low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B measurement. J Clin Lipidol. 2011;5(4):264-272.

Supplementary Table 1. Baseline characteristics

	Patients		Partners		RDD		Partners + RDD	
	MEGA n = 4463	**Tested n = 2234**	**MEGA n = 3222**	**Tested n = 1455**	**MEGA n = 2939**	**Tested n = 1418**	**MEGA n = 6161**	**Tested n = 2873**
Age, years	49 (25-68)	49 (26-67)	50 (28-66)	51 (29-66)	45 (23-67)	48 (24-67)	47 (25-66)	50 (27-67)
Women, n (%)	2442 (55)	1217 (55)	1626 (51)	746 (51)	1682 (57)	763 (54)	3308 (54)	1509 (53)
BMI (Kg/m^2)	26 (20-36)	26 (20-35)	25 (20-34)	26 (20-33)	25 (20-33)	25 (20-32)	25 (20-33)	25 (20-33)
Estrogen use, n (% in women)								
Index date	1355 (57)	726 (61)	379 (24)	179 (25)	548 (34)	227 (30)	927 (29)	406 (28)
Blood sampling	NA	205 (18)	NA	159 (23)	NA	222 (30)	NA	381 (26)
Statin use, n (%)	139 (3)	72 (3)	178 (6)	75 (5)	167 (6)	108 (8)	345 (6)	183 (6)
Self-reported diabetes, n (%)	152 (4)	72 (3)	101 (3)	46 (3)	85 (3)	41 (3)	186 (3)	87 (3)
Venous thrombosis								
DVT, n (%)	2613 (59)	1307 (59)	NA	NA	NA	NA	NA	NA
PE ± DVT, n (%)	1850 (41)	927 (41)	NA	NA	NA	NA	NA	NA

Continuous variables are shown as median (5th percentile - 95th percentile) and categorical variables as number (%).

Data were missing for some participants in some subgroups.

MEGA Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study, *RDD* random-digit dialing, *BMI* body mass index, *DVT* deep vein thrombosis, *PE* pulmonary embolism, *NA* not applicable.

Supplementary Table 2. Lipid levels according to demographic and clinical characteristics in controls

	Controls (n)	Mean level	Mean difference (95% CI)[a]	Mean difference (95% CI)[b]
TC		**(mmol L^{-1})**		
Age, years				
18 - 50	1474	5.31	reference	reference
50 - 70	1399	5.93	0.62 (0.54 to 0.70)	0.64 (0.55 to 0.72)
Sex				
Men	1364	5.59	reference	reference
Women	1509	5.63	0.05 (-0.03 to 0.12)	0.03 (-0.05 to 0.12)
Body mass index				
Normal weight	1332	5.47	reference	reference
overweight	1078	5.79	0.20 (0.12 to 0.29)	0.21 (0.13 to 0.29)
obesity	357	5.64	0.01 (-0.06 to 0.07)	0.05 (-0.01 to 0.11)
Self-reported diabetes				
No	2774	5.62	reference	reference
Yes	87	5.24	-0.65 (-0.87 to -0.42)	-0.51 (-0.74 to -0.28)
Estrogen use (women)				
Nonusers	1064	5.70	reference	reference
Users	381	5.37	0.03 (-0.09 to 0.15)	0.04 (-0.09 to 0.16)
Statin use				
Nonusers	2678	5.63	reference	reference
Users	183	5.27	-0.72 (-0.88 to -0.56)	-0.66 (-0.82 to -0.49)
LDL-C		**(mmol L^{-1})**		
Age, years				
18 - 50	1457	3.33	reference	reference
50 - 70	1371	3.79	0.46 (0.39 to 0.53)	0.47 (0.40 to 0.54)
Sex				
Men	1326	3.63	reference	reference
Women	1502	3.48	-0.14 (-0.20 to -0.07)	-0.13 (-0.20 to -0.05)
Body mass index				
Normal weight	1326	3.43	reference	reference
overweight	1050	3.72	0.19 (0.12 to 0.26)	0.19 (0.12 to 0.27)
obesity	347	3.59	0.02 (-0.03 to 0.07)	0.05 (0 to 0.11)
Self-reported diabetes				
No	2732	3.56	reference	reference
Yes	84	3.18	-0.59 (-0.78 to -0.39)	-0.45 (-0.65 to -0.25)
Estrogen use (women)				
Nonusers	1059	3.56	reference	reference
Users	380	3.22	-0.06 (-0.17 to 0.05)	-0.04 (-0.15 to 0.07)
Statin use				
Nonusers	2639	3.58	reference	reference
Users	177	3.16	-0.72 (-0.86 to -0.59)	-0.67 (-0.81 to -0.53)
Triglycerides[c]		**(mmol L^{-1})**		
Age, years				
18 - 50	1474	0.26	reference	reference
50 - 70	1399	0.41	0.15 (0.11 to 0.18)	0.10 (0.07 to 0.14)

Supplementary Table 2. (continued)

	Controls (n)	Mean level	Mean difference (95% CI)[a]	Mean difference (95% CI)[b]
Sex				
Men	1364	0.42	reference	reference
Women	1509	0.25	-0.17 (-0.20 to -0.14)	-0.19 (-0.22 to -0.15)
Body mass index				
Normal weight	1332	0.18	reference	reference
overweight	1078	0.46	0.24 (0.21 to 0.28)	0.24 (0.20 to 0.27)
obesity	357	0.53	0.16 (0.13 to 0.18)	0.16 (0.13 to 0.18)
Self-reported diabetes				
No	2774	0.32	reference	reference
Yes	87	0.66	0.27 (0.17 to 0.36)	0.16 (0.06 to 0.26)
Estrogen use (women)				
Nonusers	1064	0.24	reference	reference
Users	381	0.28	0.13 (0.08 to 0.18)	0.15 (0.10 to 0.21)
Statin use				
Nonusers	2678	0.32	reference	reference
Users	183	0.55	0.13 (0.06 to 0.20)	0.09 (0.02 to 0.16)
Apo B		**(g L^{-1})**		
Age, years				
18 - 50	1474	0.93	reference	reference
50 - 70	1399	1.05	0.12 (0.10 to 0.14)	0.12 (0.10 to 0.13)
Sex				
Men	1364	1.02	reference	reference
Women	1509	0.95	-0.07 (-0.09 to -0.06)	-0.08 (-0.10 to -0.06)
Body mass index				
Normal weight	1332	0.93	reference	reference
overweight	1078	1.04	0.07 (0.05 to 0.09)	0.08 (0.05 to 0.10)
obesity	357	1.03	0.03 (0.02 to 0.05)	0.04 (0.02 to 0.05)
Self-reported diabetes				
No	2774	0.99	reference	reference
Yes	87	0.97	-0.07 (-0.13 to -0.02)	-0.07 (-0.12 to -0.01)
Estrogen use (women)				
Nonusers	1064	0.96	reference	reference
Users	381	0.93	0.04 (0.01 to 0.07)	0.05 (0.02 to 0.08)
Statin use				
Nonusers	2678	0.99	reference	reference
Users	183	0.96	-0.11 (-0.15 to - 0.07)	-0.11 (-0.14 to -0.07)
HDL-C		**(mmol L^{-1})**		
Age, years				
18 - 50	1474	1.32	reference	reference
50 - 70	1399	1.37	0.06 (0.03 to 0.08)	0.10 (0.07 to 0.12)
Sex				
Men	1364	1.18	reference	reference
Women	1509	1.50	0.32 (0.29 to 0.34)	0.30 (0.27 to 0.32)

Supplementary Table 2. (continued)

	Controls (n)	Mean level	Mean difference (95% CI)[a]	Mean difference (95% CI)[b]
Body mass index				
Normal weight	1332	1.45	reference	reference
overweight	1078	1.26	-0.17 (-0.19 to -0.14)	-0.16 (-0.19 to -0.13)
obesity	357	1.18	-0.14 (-0.16 to -0.12)	-0.13 (-0.15 to -0.11)
Self-reported diabetes				
No	2774	1.35	reference	reference
Yes	87	1.13	-0.23 (-0.31 to -0.16)	-0.15 (-0.23 to -0.08)
Estrogen use (women)				
Nonusers	1064	1.50	reference	reference
Users	381	1.51	0.03 (-0.02 to 0.08)	0.01 (-0.04 to 0.06)
Statin use				
Nonusers	2678	1.35	reference	reference
Users	183	1.26	-0.08 (-0.13 to -0.02)	-0.04 (-0.09 to 0.02)
apo A1		**(g L^{-1})**		
Age, years				
18 - 50	1474	1.40	reference	reference
50 - 70	1399	1.46	0.06 (0.04 to 0.08)	0.09 (0.07 to 0.11)
Sex				
Men	1364	1.30	reference	reference
Women	1509	1.54	0.24 (0.22 to 0.26)	0.20 (0.18 to 0.22)
Body mass index				
Normal weight	1332	1.48	reference	reference
overweight	1078	1.38	-0.08 (-0.10 to -0.06)	-0.07 (-0.09 to -0.05)
obesity	357	1.35	-0.07 (-0.09 to -0.05)	-0.06 (-0.08 to -0.05)
Self-reported diabetes				
No	2774	1.43	reference	reference
Yes	87	1.33	-0.12 (-0.18 to -0.07)	-0.09 (-0.14 to -0.03)
Estrogen use (women)				
Nonusers	1064	1.52	reference	reference
Users	381	1.61	0.13 (0.09 to 0.16)	0.12 (0.08 to 0.15)
Statin use				
Nonusers	2678	1.43	reference	reference
Users	183	1.42	-0.01 (-0.05 to 0.03)	0.01 (-0.03 to 0.05)

Data were missing for some participants in some subgroups.
Estrogen use was determined at the time of blood sampling.
Normal weight was defined as a body mass index 18.5-25 kg/m^2, overweight as a body mass index 25-30 kg/m^2, and obesity as a body mass index >30 kg/m^2.
TC total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *Apo B* apolipoprotein B, *HDL-C* high-density lipoprotein cholesterol, *Apo A1* apolipoprotein A1.
[a]Mean difference adjusted for age and sex (where applicable).
[b]Mean difference adjusted for age, sex, body mass index, self-reported diabetes, estrogen use at blood sampling, and statin use (where applicable).
[c]Log-transformed.

Chapter 3

Lipid levels and risk of recurrent venous thrombosis: results from the MEGA follow-up study

V.M. Morelli, W.M. Lijfering, F.R. Rosendaal, S.C. Cannegieter

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ABSTRACT

Background

Knowledge of risk factors for recurrent venous thrombosis may guide decisions on duration of anticoagulation. The association between lipid levels and first venous thrombosis has been studied extensively. However, data on the role of lipids in the risk of recurrence are scarce.

Objective

To assess the association between lipid levels and recurrent venous thrombosis.

Patients/Methods

Patients with a first venous thrombosis were included from the MEGA study. Follow-up started at the date of end of anticoagulant treatment. Percentile categories of total/low-density lipoprotein/high-density lipoprotein cholesterol, triglycerides, and apolipoproteins B and A1 were established (<10^{th}, 10^{th}-25^{th}, 25^{th}-75^{th} [reference], 75^{th}-90^{th}, >90^{th} percentile). Lipids were measured at least 3 months after discontinuing anticoagulation.

Results

Of 2106 patients followed for a median of 6.9 years, 326 developed recurrence (incidence rate 2.7/100 patient-years; 95% confidence interval [CI] 2.5-3.1). With hazard ratios ranging from 0.88 (95%CI 0.55-1.42) to 1.33 (95%CI 0.86-2.04) in the highest percentile category vs the reference, we found no association across percentile categories between recurrence and lipid levels in age- and sex-adjusted models, nor after further adjustments for body mass index, diabetes, estrogen- and statin-use, and duration of anticoagulation. Subgroup analyses stratified by unprovoked or provoked first events, location (deep vein thrombosis or pulmonary embolism), and sex neither revealed an association with any of the lipid levels studied.

Conclusions

Testing lipid levels did not identify patients at an increased risk of recurrent venous thrombosis in this study, including those with unprovoked first events, and these should not influence decisions on duration of anticoagulation.

INTRODUCTION

Venous thrombosis (VT) is a common disease [1] with a high 5-year cumulative incidence of recurrence that varies among studies from 12% to 30% [2,3]. Clinicians and patients often face a dilemma, in which discontinuing anticoagulant treatment may result in a new thrombotic event, while continuing oral anticoagulation is accompanied with an incidence of major bleeding of 1-3% per year [4,5]. In this respect, knowledge of risk factors for recurrent VT is crucial, as it may guide decisions on duration of anticoagulation after a first event of VT.

Several studies have suggested that lipid-lowering drugs (i.e., statins) are associated with a decreased risk of VT, including recurrence [6-8]. Such findings make lipids interesting candidates to be assessed in relation to the risk of VT. Indeed, there has been an increasing number of studies exploring the effect of lipid levels on the risk of a first event of VT in the past few years [9-16], which results have been controversial, particularly regarding the effect of apolipoproteins [10,11,14,15]. In contrast, data on the role of lipid levels in the risk of recurrent VT are scarce [17-19]. Hence, whether testing for lipids identifies patients at an increased risk of a recurrent event is as yet unclear.

Therefore, we aimed to investigate the association between lipid levels and risk of recurrent VT in a follow-up study, with particular attention to subgroup analyses, as patients with unprovoked first events and men are at an increased risk of recurrence [2,3]. For this purpose, we used data from the Multiple Environmental and Genetic Assessment of risk factors for VT (MEGA) follow-up study. We evaluated total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, and apolipoproteins A1 and B.

METHODS

Patients

Patients were recruited from the MEGA study, which details have been described elsewhere [20,21]. Briefly, between March 1999 and August 2004, 4956 patients aged 18-70 years with a first deep vein thrombosis (DVT) of the leg, pulmonary embolism (PE) or both were included. Of these, 225 did not consent for follow-up, leaving 4731 patients for the MEGA follow-up study. In the current analyses, 456 patients with active or previous history of malignancy within 5 years before first event were excluded. For logistic reasons, patients were asked to provide blood samples till June 2002 only. Among the 4275 patients eligible for follow-up, 2215 provided blood samples. Lastly, 109 patients were on anticoagulant treatment at the end of follow-up and were excluded, leaving 2106 patients with follow-up starting at the date of discontinuation of anticoagulation. Between 2007 and 2009, the vital status of all

patients was acquired from the central Dutch population register, and causes of death were obtained from the national register of death certificates at the Central Bureau of Statistics [21,22].This study was approved by the Ethics Committee of the Leiden University Medical Center, and all participants gave written informed consent.

Initial questionnaire (baseline characteristics) and blood sampling

Patients filled in a questionnaire on potential risk factors for VT within a few weeks after registration at the anticoagulation clinic [20]. Of interest for this analysis are body weight and height, estrogen- and statin-use, and self-reported diabetes. Body mass index (BMI) was calculated by dividing weight (in kg) by height squared (m^2). The index date was the date of diagnosis of the first thrombotic event. Unprovoked first VT was defined in the absence of trauma, surgery, immobilization (bedridden at home or hospitalization), plaster cast or pregnancy in the first 3 months before the index date, long-distance travel in the first 2 months before the index date, or estrogen use (oral contraceptive or hormonal replacement therapy) at the index date. Blood sampling was obtained at least 3 months after discontinuing anticoagulation, or 1 year after the index date in case of prolonged anticoagulation.

Assessment of recurrent VT

Short questionnaires concerning recurrence were sent to all consenting patients known to be alive between June 2008 and July 2009 [21]. Additional information was obtained from regional anticoagulation clinics and hospitals. Deaths owing to recurrent VT were considered fatal recurrent events. Based on hospital discharge letters, information from the anticoagulation clinics, questionnaires and causes of death, possible recurrences were classified into certain and uncertain events using a decision rule as previously described [21].

Laboratory measurements

Lipid levels were measured on stored (-80°C) and previously unthawed fasting serum samples. TC and triglycerides were measured by a colorimetric method on a Modular P analyser (Roche Diagnostics, Mannheim, Germany). HDL-C was measured by a direct method based on the Kyowa Medex reaction principle using polyethylene glycol (PEG)-modified enzymes (Roche Diagnostics, Mannheim, Germany). Apolipoproteins A1 and B were measured by immunoturbidimetry on a Cobas Integra analyzer (Roche Diagnostics, Mannheim, Germany). LDL-C levels were estimated using the Friedewald formula [23], and when triglycerides exceeded 4.52 mmol/L, LDL-C was not estimated.

Statistical analysis

Duration of follow-up was defined as the time from the date of discontinuation of anticoagulation to the end of follow-up, which was defined as the date of recurrence, death or emigration, or date of filling in the short questionnaire. If patients did not complete the questionnaire, they were censored at the last date we knew them to be recurrence-free [21] (date of death [n=17], date of emigration [n=1], or date when they were last seen by the anticoagulation clinic or for research purposes [n=246]). Analyses were limited to certain recurrences (n=326), and patients with uncertain recurrent events (n=77) were censored at that time.

Lipid categories were defined *a priori* according to the levels measured in controls from the MEGA study (<10th, 10th-25th, 25th-75th [reference category], 75th-90th, and >90th percentile) [24]. Crude incidence rates with 95% confidence intervals (CIs) of recurrent VT were estimated as the number of events over the accumulated follow-up time. Cox proportional hazard regression models were used to obtain hazard ratios (HRs) for recurrence with 95% CIs. HRs were adjusted for age and sex, estrogen use at blood sampling (dichotomous value), BMI (continuous values), statin use (dichotomous value), self-reported diabetes (dichotomous value), and duration of anticoagulant treatment (dichotomized as <6 months and ≥6 months). The proportional hazard assumption was verified by evaluating the parallelism between the curves of the log-log survivor function.

Subgroup analyses involved stratification by VT type (unprovoked or provoked first events), VT initial location (DVT or PE), and sex. To quantify potential misclassification of outcomes, we performed several sensitivity analyses for overall recurrence: follow-up started at the date of first event or blood sampling, certain and uncertain recurrences were both taken into account, and patients lost to follow-up were considered to have developed recurrence at the end of the study (for which date of recurrence was set at the date on which vital status was checked). Statistical analyses were performed with SPSS for Windows, release 20.0 (SPSS Inc, Chicago, IL).

RESULTS AND DISCUSSION

Median duration of follow-up was 6.9 years (interquartile range [IQR] 2.9-8.0 years) among the 2106 patients with a first event of VT. Table 1 shows no substantial differences in the baseline characteristics of all patients compared with those who provided blood samples, indicating that the tested patients were representative of the whole cohort eligible for follow-up. Median age at discontinuation of anticoagulation was 49 years, 1161 (55%) patients were women, and most first events were provoked (70%) and DVTs (59%). Median time between first event and discontinuation of anticoagulation was 6 months (IQR 3.5-6.9 months).

Table 1. Baseline characteristics

	MEGA follow-up cohort*	Included for analyses
Total	4275 (100)	2106 (100)
Women	2351 (55)	1161 (55)
Age at discontinuation of anticoagulant therapy (years)	49 (38-59)	49 (38-58)
Classical venous thrombosis risk factors		
Provoked by†:	2876 (69)	1456 (70)
Trauma/surgery/immobilization	1661 (58)	824 (57)
Plaster cast	214 (7)	108 (7)
Estrogen use (women)	1322 (46)	696 (48)
Pregnancy/puerperium (women)	171 (6)	91 (6)
Travel > 4 h	689 (24)	364 (25)
Unprovoked	1301 (31)	621 (30)
Type of index event		
Deep vein thrombosis only	2497 (58)	1250 (59)
Pulmonary embolism ± deep vein thrombosis	1778 (42)	856 (41)

Continuous variables are shown as median (25th - 75th percentiles) and categorical variables as number (%).

Data were missing for some participants in some subgroups.

MEGA, Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis.

*Patients with active or previous history of malignancy 5 years before the first event were excluded from current analyses.

†As concomitance of provoked risk factors occurred frequently, patients could be counted twice or more.

During follow-up (11,900 patient-years), 326 patients developed recurrent VT, yielding an overall incidence rate of 2.7/100 patient-years (95% CI 2.5-3.1). Table 2 shows that TC, LDL-C, triglycerides, HDL-C, and apolipoproteins B and A1 levels were not associated with an increased risk of recurrence across percentile categories in age- and sex-adjusted models and after full adjustment. Likewise, sensitivity analyses revealed no association between lipid levels and risk of recurrence (data not shown).

As expected, rates of recurrence were higher in patients with unprovoked first events (4.5/100 patient-years, 95% CI 3.8-5.3) and men (4.1/100 patient-years, 95% CI 3.5-4.7) compared with those with provoked first events (2.1/100 patient-years, 95% CI 1.8-2.4) and women (1.8/100 patient-years, 95% CI 1.5-2.1). Subgroup analyses stratified by unprovoked or provoked first events, VT initial location (DVT or PE), and sex showed no consistent association across percentile categories between lipid levels and recurrence (Tables 3 and 4).

In this study, levels of TC, LDL-C, triglycerides, HDL-C and apolipoproteins B and apo A1 were not associated with an increased risk of recurrent VT, and none of these lipids appeared to influence the risk of recurrence in specific groups. Moreover, on the basis of our results, in the hypothesis that statins are causally associated with

Table 2. Lipid levels and risk of recurrent venous thrombosis

	PY	Events	IR	(95%CI)	HR*	(95%CI)	HR†	(95%CI)
TC (mmol L^{-1})								
< 10th (<4.28)	1133	29	2.56	(1.71-3.68)	1.03	(0.69-1.53)	1.07	(0.71-1.62)
10th - 25th (4.28-4.84)	1888	34	1.80	(1.25-2.52)	0.72	(0.49-1.04)	0.77	(0.53-1.12)
25th - 75th (4.84-6.30)	6089	174	2.86	(2.45-3.32)	1	reference	1	reference
75th - 90th (6.30-7.04)	1681	48	2.86	(2.11-3.79)	0.95	(0.69-1.31)	0.95	(0.68-1.33)
> 90th (>7.04)	1109	41	3.70	(2.65-5.02)	1.23	(0.87-1.72)	1.14	(0.79-1.64)
LDL-C (mmol L^{-1})								
< 10th (<2.38)	1119	27	2.41	(1.59-3.51)	1.07	(0.71-1.61)	1.12	(0.73-1.70)
10th - 25th (2.38-2.87)	1684	38	2.26	(1.60-3.09)	1.00	(0.70-1.44)	0.98	(0.68-1.42)
25th - 75th (2.87-4.17)	6075	158	2.60	(2.21-3.04)	1	reference	1	reference
75th - 90th (4.17-4.85)	1783	66	3.70	(2.86-4.71)	1.29	(0.97-1.73)	1.20	(0.88-1.62)
> 90th (>4.85)	1125	32	2.84	(1.95-4.02)	1.01	(0.69-1.48)	0.96	(0.65-1.44)
Triglycerides (mmol L^{-1})								
< 10th (<0.79)	1200	20	1.67	(1.02-2.57)	0.73	(0.46-1.18)	0.74	(0.46-1.21)
10th - 25th (0.79-1.00)	1812	33	1.82	(1.25-2.56)	0.76	(0.52-1.10)	0.75	(0.51-1.12)
25th - 75th (1.00-1.88)	6033	166	2.75	(2.35-3.20)	1	reference	1	reference
75th - 90th (1.88-2.58)	1736	69	3.97	(3.09-5.03)	1.29	(0.97-1.71)	1.23	(0.92-1.65)
> 90th (>2.58)	1119	38	3.40	(2.40-4.66)	1.02	(0.72-1.46)	1.01	(0.70-1.45)
Apo B (g L^{-1})								
< 10th (<0.68)	1666	29	1.74	(1.17-2.50)	0.76	(0.51-1.13)	0.82	(0.54-1.25)
10th - 25th (0.68-0.80)	1950	42	2.15	(1.55-2.91)	0.86	(0.61-1.21)	0.85	(0.59-1.21)
25th - 75th (0.80-1.15)	5815	170	2.92	(2.50-3.40)	1	reference	1	reference
75th - 90th (1.15-1.33)	1609	52	3.23	(2.41-4.24)	0.99	(0.72-1.35)	1.03	(0.74-1.41)
> 90th (>1.33)	860	33	3.83	(2.64-5.39)	1.19	(0.82-1.73)	1.16	(0.79-1.70)
HDL-C (mmol L^{-1})								
< 10th (<0.90)	1252	49	3.91	(2.90-5.17)	1.11	(0.80-1.54)	1.08	(0.77-1.53)
10th - 25th (0.90-1.07)	2050	56	2.73	(2.06-3.55)	0.84	(0.62-1.15)	0.82	(0.60-1.12)
25th - 75th (1.07-1.56)	5705	157	2.75	(2.34-3.22)	1	reference	1	reference
75th - 90th (1.56-1.86)	1868	44	2.36	(1.71-3.16)	1.01	(0.72-1.41)	1.06	(0.75-1.50)
> 90th (>1.86)	1025	20	1.95	(1.19-3.01)	0.88	(0.55-1.42)	0.70	(0.41-1.20)
Apo A1 (g L^{-1})								
< 10th (<1.09)	1603	51	3.18	(2.37-4.18)	1.04	(0.75-1.44)	1.02	(0.73-1.42)
10th - 25th (1.09-1.22)	1725	56	3.25	(2.45-4.21)	1.16	(0.85-1.57)	1.11	(0.81-1.53)
25th - 75th (1.22-1.59)	5996	153	2.55	(2.16-2.98)	1	reference	1	reference
75th - 90th (1.59-1.81)	1672	41	2.45	(1.76-3.33)	1.13	(0.80-1.61)	1.06	(0.73-1.54)
> 90th (>1.81)	904	25	2.76	(1.79-4.08)	1.33	(0.86-2.04)	1.16	(0.73-1.84)

Lipid categories were defined according to the levels measured in the control group from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis (MEGA) Study [24].
Data were missing for some participants in some subgroups.
apo A1, apolipoprotein A1; apo B, apolipoprotein B; CI, confidence interval; HR, hazard ratio; HDL-C, high-density lipoprotein cholesterol; IR, incidence rate per 100 patient-years; LDL-C, low-density lipoprotein cholesterol; PY, patient-years; TC, total cholesterol.
*Adjusted for age and sex.
†Adjusted for age, sex, body mass index, estrogen use at blood sampling, statin use, self-reported diabetes, and duration of anticoagulant treatment.

Table 3. Lipid levels and risk of recurrent venous thrombosis by type and location of first event

	Unprovoked venous thrombosis n = 621				Provoked venous thrombosis n = 1456				Deep vein thrombosis n = 1250				Pulmonary embolism‡ n = 856			
	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)
TC (mmol L^{-1})																
< 10th (<4.28)	1.44	(0.80-2.61)	1.47	(0.81-2.67)	0.80	(0.47-1.38)	0.87	(0.49-1.54)	1.17	(0.71-1.93)	1.17	(0.70-1.97)	0.86	(0.44-1.68)	0.92	(0.46-1.85)
10th - 25th (4.28-4.84)	0.85	(0.49-1.46)	0.86	(0.50-1.50)	0.60	(0.36-1.00)	0.69	(0.41-1.15)	0.71	(0.44-1.15)	0.75	(0.46-1.21)	0.74	(0.41-1.33)	0.81	(0.45-1.47)
25th - 75th (4.84-6.30)	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference
75th - 90th (6.30-7.04)	1.15	(0.72-1.85)	1.05	(0.64-1.71)	0.85	(0.54-1.32)	0.90	(0.57-1.42)	0.99	(0.66-1.47)	1.04	(0.69-1.56)	0.88	(0.51-1.53)	0.81	(0.45-1.45)
> 90th (>7.04)	1.37	(0.86-2.17)	1.22	(0.74-2.00)	1.02	(0.61-1.71)	0.99	(0.58-1.68)	1.20	(0.79-1.83)	1.14	(0.74-1.77)	1.27	(0.71-2.26)	1.08	(0.56-2.05)
LDL-C (mmol L^{-1})																
< 10th (<2.38)	1.31	(0.69-2.48)	1.34	(0.70-2.59)	0.94	(0.55-1.62)	1.04	(0.60-1.80)	1.24	(0.76-2.04)	1.27	(0.77-2.10)	0.82	(0.39-1.74)	0.83	(0.38-1.80)
10th - 25th (2.38-2.87)	1.18	(0.68-2.04)	1.21	(0.69-2.10)	0.87	(0.54-1.40)	0.82	(0.50-1.36)	0.92	(0.59-1.46)	0.86	(0.54-1.38)	1.16	(0.65-2.07)	1.18	(0.65-2.16)
25th - 75th (2.87-4.17)	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference
75th - 90th (4.17-4.85)	1.85	(1.22-2.80)	1.71	(1.11-2.63)	1.02	(0.67-1.53)	0.93	(0.61-1.43)	1.21	(0.83-1.76)	1.13	(0.76-1.66)	1.45	(0.92-2.30)	1.33	(0.83-2.15)
> 90th (>4.85)	1.31	(0.80-2.15)	1.18	(0.70-2.00)	0.69	(0.37-1.29)	0.70	(0.37-1.31)	0.94	(0.59-1.49)	0.91	(0.57-1.47)	1.14	(0.58-2.25)	0.98	(0.46-2.08)

Table 3. (continued)

	Unprovoked venous thrombosis n = 621				Provoked venous thrombosis n = 1456				Deep vein thrombosis n = 1250				Pulmonary embolism‡ n = 856			
	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)
Triglycerides (mmol L^{-1})																
< 10th (<0.79)	0.97	(0.39-2.41)	0.94	(0.37-2.35)	0.67	(0.39-1.17)	0.71	(0.40-1.27)	0.74	(0.42-1.30)	0.77	(0.43-1.37)	0.70	(0.30-1.64)	0.65	(0.26-1.67)
10th - 25th (0.79-1.00)	0.83	(0.45-1.53)	0.80	(0.42-1.52)	0.68	(0.42-1.10)	0.73	(0.44-1.19)	0.84	(0.53-1.32)	0.81	(0.50-1.30)	0.62	(0.32-1.22)	0.63	(0.31-1.29)
25th - 75th (1.00-1.88)	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.88-2.58)	1.86	(1.26-2.73)	1.77	(1.19-2.64)	0.87	(0.56-1.35)	0.82	(0.52-1.28)	1.09	(0.75-1.57)	1.09	(0.74-1.58)	1.67	(1.07-2.60)	1.48	(0.92-2.38)
> 90th (>2.58)	1.15	(0.71-1.87)	1.22	(0.74-2.02)	0.89	(0.52-1.52)	0.81	(0.46-1.41)	1.08	(0.70-1.65)	1.12	(0.72-1.73)	0.89	(0.47-1.70)	0.79	(0.40-1.57)
Apo B (g L^{-1})																
< 10th (<0.68)	0.94	(0.50-1.77)	0.97	(0.51-1.87)	0.67	(0.40-1.13)	0.76	(0.44-1.32)	0.89	(0.54-1.49)	0.95	(0.57-1.57)	0.59	(0.29-1.20)	0.60	(0.28-1.30)
10th - 25th (0.68-0.80)	0.78	(0.44-1.38)	0.81	(0.45-1.46)	0.91	(0.59-1.40)	0.88	(0.55-1.39)	0.89	(0.57-1.37)	0.81	(0.50-1.29)	0.83	(0.48-1.44)	0.90	(0.51-1.59)
25th - 75th (0.80-1.15)	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.15-1.33)	1.13	(0.73-1.76)	1.13	(0.72-1.78)	0.92	(0.59-1.43)	0.93	(0.59-1.47)	0.89	(0.60-1.32)	0.93	(0.62-1.39)	1.17	(0.70-1.94)	1.18	(0.69-2.01)
> 90th (>1.33)	1.45	(0.89-2.36)	1.41	(0.85-2.35)	0.86	(0.47-1.58)	0.86	(0.47-1.58)	1.16	(0.73-1.84)	1.11	(0.69-1.78)	1.23	(0.65-2.33)	1.17	(0.59-2.31)

Table 3. (continued)

	Unprovoked venous thrombosis n = 621				Provoked venous thrombosis n = 1456				Deep vein thrombosis n = 1250				Pulmonary embolism‡ n = 856			
	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)	HR*	(95%CI)	HR†	(95%CI)
HDL-C (mmol L^{-1})																
< 10th (<0.90)	1.34	(0.86-2.08)	1.44	(0.90-2.30)	0.85	(0.51-1.41)	0.77	(0.45-1.32)	0.93	(0.61-1.41)	0.97	(0.62-1.49)	1.50	(0.89-2.53)	1.33	(0.76-2.33)
10th - 25th (0.90-1.07)	1.00	(0.66-1.52)	1.04	(0.68-1.61)	0.64	(0.40-1.03)	0.59	(0.36-0.96)	0.73	(0.49-1.10)	0.71	(0.47-1.07)	1.06	(0.65-1.72)	1.04	(0.63-1.71)
25th - 75th (1.07-1.56)	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.56-1.86)	0.74	(0.39-1.42)	0.77	(0.39-1.52)	1.14	(0.76-1.70)	1.23	(0.81-1.85)	1.11	(0.74-1.66)	1.17	(0.77-1.76)	0.80	(0.43-1.50)	0.84	(0.43-1.61)
> 90th (>1.86)	1.22	(0.60-2.48)	1.10	(0.52-2.37)	0.66	(0.34-1.28)	0.49	(0.22-1.06)	0.90	(0.51-1.58)	0.73	(0.39-1.38)	0.85	(0.36-2.00)	0.63	(0.22-1.78)
Apo A1 (g L^{-1})																
< 10th (<1.09)	1.20	(0.76-1.89)	1.25	(0.78-2.00)	0.90	(0.57-1.43)	0.85	(0.52-1.37)	0.81	(0.53-1.23)	0.83	(0.54-1.28)	1.59	(0.96-2.64)	1.48	(0.87-2.51)
10th - 25th (1.09-1.22)	1.58	(1.05-2.39)	1.65	(1.07-2.53)	0.75	(0.46-1.22)	0.72	(0.43-1.18)	0.90	(0.60-1.36)	0.86	(0.57-1.31)	1.75	(1.08-2.83)	1.78	(1.08-2.92)
25th - 75th (1.22-1.59)	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.59-1.81)	1.08	(0.60-1.93)	1.10	(0.60-2.01)	1.17	(0.75-1.84)	1.07	(0.67-1.72)	1.09	(0.71-1.67)	1.00	(0.63-1.58)	1.21	(0.65-2.25)	1.23	(0.64-2.36)
> 90th (>1.81)	1.37	(0.70-2.71)	1.18	(0.56-2.52)	1.25	(0.72-2.18)	1.08	(0.60-1.93)	1.16	(0.68-1.97)	1.01	(0.58-1.78)	1.75	(0.85-3.62)	1.53	(0.67-3.47)

Lipid categories were defined according to the levels measured in the control group from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis (MEGA) Study [24]. Data were missing for some participants in some subgroups. apo A1, apolipoprotein A1; apo B, apolipoprotein B; CI, confidence interval; HR, hazard ratio; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol. *Adjusted for age and sex.†Adjusted for age, sex, body mass index, estrogen use at blood sampling, statin use, self-reported diabetes, and duration of anticoagulant treatment. ‡Pulmonary embolism with or without symptomatic deep vein thrombosis.

Table 4. Lipid levels and risk of recurrent venous thrombosis by sex

	Men n = 943				Women n = 1161			
	HR*	**(95%CI)**	**HR†**	**(95%CI)**	**HR***	**(95%CI)**	**HR†**	**(95%CI)**
TC (mmol L^{-1})								
< 10th (<4.28)	1.23	(0.73-2.05)	1.15	(0.67-1.99)	0.78	(0.41-1.47)	0.96	(0.50-1.83)
10th - 25th (4.28-4.84)	0.89	(0.56-1.43)	0.92	(0.58-1.48)	0.51	(0.28-0.93)	0.61	(0.33-1.13)
25th - 75th (4.84-6.30)	1	reference	1	reference	1	reference	1	reference
75th - 90th (6.30-7.04)	0.91	(0.60-1.37)	0.82	(0.53-1.26)	1.05	(0.62-1.78)	1.26	(0.74-2.15)
> 90th (>7.04)	1.28	(0.84-1.93)	1.18	(0.78-1.81)	1.19	(0.65-2.18)	1.04	(0.51-2.13)
LDL-C (mmol L^{-1})								
< 10th (<2.38)	1.27	(0.74-2.19)	1.23	(0.70-2.16)	0.84	(0.45-1.56)	0.94	(0.50-1.79)
10th - 25th (2.38-2.87)	1.41	(0.90-2.21)	1.38	(0.87-2.18)	0.63	(0.35-.1.13)	0.61	(0.33-1.12)
25th - 75th (2.87-4.17)	1	reference	1	reference	1	reference	1	reference
75th - 90th (4.17-4.85)	1.31	(0.92-1.88)	1.21	(0.84-1.75)	1.31	(0.80-2.15)	1.19	(0.70-2.02)
> 90th (>4.85)	1.16	(0.74-1.82)	1.06	(0.67-1.68)	0.77	(0.36-1.62)	0.74	(0.32-1.73)
Triglycerides (mmol L^{-1})								
< 10th (<0.79)	0.91	(0.46-1.80)	0.95	(0.47-1.89)	0.60	(0.31-1.15)	0.60	(0.30-1.18)
10th - 25th (0.79-1.00)	0.98	(0.60-1.61)	0.99	(0.59-1.64)	0.57	(0.31-0.99)	0.54	(0.29-1.02)
25th - 75th (1.00-1.88)	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.88-2.58)	1.33	(0.95-1.87)	1.31	(0.92-1.86)	1.24	(0.74-2.09)	1.09	(0.62-1.90)
> 90th (>2.58)	0.95	(0.63-1.45)	0.95	(0.62-1.47)	1.41	(0.72-2.75)	1.33	(0.65-2.72)
Apo B (g L^{-1})								
< 10th (<0.68)	0.98	(0.56-1.72)	0.99	(0.56-1.75)	0.60	(0.33-1.07)	0.69	(0.37-1.28)
10th - 25th (0.68-0.80)	0.88	(0.55-1.42)	0.93	(0.56-1.52)	0.81	(0.50-1.33)	0.80	(0.47-1.37)
25th - 75th (0.80-1.15)	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.15-1.33)	0.95	(0.66-1.38)	0.96	(0.66-1.40)	1.13	(0.63-2.02)	1.25	(0.68-2.31)
> 90th (>1.33)	1.21	(0.78-1.89)	1.15	(0.73-1.81)	1.18	(0.58-2.38)	1.18	(0.56-2.49)
HDL-C (mmol L^{-1})								
< 10th (<0.90)	1.07	(0.74-1.55)	1.10	(0.74-1.61)	1.40	(0.67-2.92)	1.16	(0.51-2.60)
10th - 25th (0.90-1.07)	0.83	(0.58-1.19)	0.85	(0.59-1.22)	0.92	(0.50-1.66)	0.79	(0.42-1.52)
25th - 75th (1.07-1.56)	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.56-1.86)	1.27	(0.80-2.02)	1.29	(0.81-2.06)	0.83	(0.51-1.34)	0.88	(0.53-1.46)
> 90th (>1.86)	0.75	(0.30-1.83)	0.61	(0.22-1.68)	0.94	(0.53-1.65)	0.74	(0.38-1.41)
Apo A1 (g L^{-1})								
< 10th (<1.09)	0.98	(0.67-1.41)	0.99	(0.68-1.45)	1.34	(0.70-2.57)	1.21	(0.61-2.42)
10th - 25th (1.09-1.22)	1.14	(0.79-1.64)	1.17	(0.81-1.69)	1.20	(0.68-2.13)	1.09	(0.59-2.01)
25th - 75th (1.22-1.59)	1	reference	1	reference	1	reference	1	reference
75th - 90th (1.59-1.81)	1.25	(0.76-2.05)	1.22	(0.73-2.05)	1.05	(0.64-1.73)	0.94	(0.55-1.61)
> 90th (>1.81)	1.20	(0.58-2.47)	1.25	(0.61-2.58)	1.42	(0.82-2.45)	1.13	(0.62-2.07)

Lipid categories were defined according to the levels measured in the control group from the Multiple Environmental and Genetic Assessment of Risk Factors for Venous Thrombosis (MEGA) Study [24]. Data were missing for some participants in some subgroups. apo A1, apolipoprotein A1; apo B, apolipoprotein B; CI, confidence interval; HR, hazard ratio; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol. *Adjusted for age. †Ajusted for age, body mass index, estrogen use at blood sampling (women), statin use, self-reported diabetes, and duration of anticoagulant treatment.

a decreased risk of recurrent VT, it is unlikely that this effect is due to statin lipid-lowering activities.

Only a few cohort studies have addressed the role of lipid levels in the risk of recurrent VT till date, and they were heterogeneous regarding the lipids studied [17-19]. In the Austrian Study on Recurrent Venous Thromboembolism [17], 772 patients with first unprovoked VT were followed for a median of 48 months. High apolipoprotein A1 levels were associated with a decreased risk of recurrence, whereas apolipoprotein B levels after adjustment for age and sex had no effect on the risk of recurrence. In contrast, we found no association between apolipoprotein A1 levels and recurrence, even among patients with unprovoked first events. The source and selection criteria of the study population, and the duration of follow-up might have accounted for the differences between the two cohorts. A Canadian study of 510 patients with first unprovoked VT [18], followed for a mean of 16.9 months, found no association between lipoprotein (a) levels and recurrence. Finally, a Swedish study of 443 patients with first unprovoked VT [19], followed for a mean of 36 months, found that low apolipoprotein M levels appeared to increase the risk of recurrence but in men only.

The strengths of this study include that this is the largest population-based cohort study that has been performed on this issue so far, in which levels of several lipids were measured after a first event of VT. Patients were followed for a long period of time for a recurrent event that was objectively confirmed [21]. Furthermore, several subgroup analyses were performed, enabling us to obtain detailed risk estimates. Some limitations of the study need to be addressed. First, weak associations between lipid levels and recurrence might have been missed due to inadequate statistical power in some subgroups, reflected in the wide CIs of the point estimates. However, weak associations, if present, will have little to no consequence regarding clinical decision-making [25]. Second, owing to the design of cohort studies, there was a time lag between the exposure (lipid assessment) and outcome (recurrence). Lipid levels might have changed over time in both groups, with and without recurrence, due to prescription of lipid-lowering drugs, changes in lifestyle (i.e., diet and physical activity), or simply aging, which could have resulted in an underestimation of the effect of lipid levels on the risk of recurrence. Third, patients were included in this study if they experienced their first event of VT up to 70 years old, and our results may therefore not be generalizable to an elderly population (i.e., >70 years old). Fourth, as blood was collected after the first event of VT, lipid levels might have been affected by acute-phase reactions at the time of the first event [26,27]. However, to avoid this problem blood was drawn with a median of 10 months (IQR 8.3-12.1 months) after the thrombotic event, by which time the effects of the acute-phase reaction would have worn off [28,29].

In conclusion, we have assessed the role of lipid levels in the risk of recurrent VT in a large longitudinal cohort of patients with a first VT, and found no evidence of an association, even among men or those with a first unprovoked event. Testing for lipid levels does not appear to be useful to identify patients at an increased risk of recurrence, and should not influence clinical decision-making regarding VT treatment.

REFERENCES

1. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrøm J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 2007; 5: 692-99.
2. Christiansen SC, Cannegieter SC, Koster T, Vandenbroucke JP, Rosendaal FR. Thrombophilia, clinical factors, and recurrent venous thrombotic events. JAMA 2005; 293: 2352-61.
3. Prandoni P, Noventa F, Ghirarduzzi A, Pengo V, Bernardi E, Pesavento R, Iotti M, Tormene D, Simioni P, Pagnan A. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. Haematologica 2007; 92: 199-205.
4. Veeger NJ, Piersma-Wichers M, Tijssen JG, Hillege HL, Van Der Meer J. Individual time within target range in patients treated with vitamin K antagonists: main determinant of quality of anticoagulation and predictor of clinical outcome. A retrospective study of 2300 consecutive patients with venous thromboembolism. Br J Haematol 2005; 128: 513-19.
5. Kearon C, Gent M, Hirsh J, Weitz J, Kovacs MJ, Anderson DR, Turpie AG, Green D, Ginsberg JS, Wells P, MacKinnon B, Julian JA. A comparison of three months of anticoagulation with extended anticoagulation for a first episode of idiopathic venous thromboembolism. N Engl J Med 1999; 340: 901-7.
6. Lijfering WM, Biedermann JS, Kruip MJ, Leebeek FW, Rosendaal FR, Cannegieter SC. Can we prevent venous thrombosis with statins: an epidemiologic review into mechanism and clinical utility. Expert Rev Hematol 2016; 9: 1023-30.
7. Biere-Rafi S, Hutten BA, Squizzato A, Ageno W, Souverein PC, de Boer A, Gerdes VE, Büller HR, Kamphuisen PW. Statin treatment and the risk of recurrent pulmonary embolism. Eur Heart J 2013; 34: 1800-6.
8. Schmidt M, Cannegieter SC, Johannesdottir SA, Dekkers OM, Horváth-Puhó E, Sørensen HT. Statin use and venous thromboembolism recurrence: a combined nationwide cohort and nested case-control study. J Thromb Haemost 2014; 12: 1207-15.
9. Braekkan SK, Mathiesen EB, Njølstad I, Wilsgaard T, Størmer J, Hansen JB. Family history of myocardial infarction is an independent risk factor for venous thromboembolism: the Tromsø study. J Thromb Haemost 2008; 6: 1851-57.
10. Chamberlain AM, Folsom AR, Heckbert SR, Rosamond WD, Cushman M. High-density lipoprotein cholesterol and venous thromboembolism in the Longitudinal Investigation of Thromboembolism Etiology (LITE). Blood 2008; 112: 2675-80.
11. Everett BM, Glynn RJ, Buring JE, Ridker PM. Lipid biomarkers, hormone therapy and the risk of venous thromboembolism in women. J Thromb Haemost 2009; 7: 588-96.
12. Quist-Paulsen P, Naess IA, Cannegieter SC, Romundstad PR, Christiansen SC, Rosendaal FR, Hammerstrøm J. Arterial cardiovascular risk factors and venous thrombosis: results from a population-based, prospective study (the HUNT 2). Haematologica 2010; 95: 119-25.

13. Holst AG, Jensen G, Prescott E. Risk factors for venous thromboembolism: results from the Copenhagen City Heart Study. Circulation 2010; 121: 1896-903.
14. van Schouwenburg IM, Mahmoodi BK, Gansevoort RT, Muntinghe FL, Dullaart RP, Kluin Nelemans HC, Veeger NJ, Meijer K. Lipid levels do not influence the risk of venous thromboembolism. Results of a population-based cohort study. Thromb Haemost 2012; 108: 923-9.
15. Delluc A, Malécot JM, Kerspern H, Nowak E, Carre JL, Mottier D, Le Gal G, Lacut K. Lipid parameters, lipid lowering drugs and the risk of venous thromboembolism. Atherosclerosis 2012; 220: 184-8.
16. Wattanakit K, Lutsey PL, Bell EJ, Gornik H, Cushman M, Heckbert SR, Rosamond WD, Folsom AR. Association between cardiovascular disease risk factors and occurrence of venous thromboembolism. A time-dependent analysis. Thromb Haemost 2012; 108: 508-15.
17. Eichinger S, Pecheniuk NM, Hron G, Deguchi H, Schemper M, Kyrle PA, Griffin JH. High-density lipoprotein and the risk of recurrent venous thromboembolism. Circulation 2007; 115: 1609-14.
18. Rodger MA, Le Gal G, Carrier M, Betancourt MT, Kahn SR, Wells PS, Anderson DA, Lacut K, Chagnon I, Solymoss S, Crowther M, Perrier A, White R, Vickars L, Ramsay T, Kovacs MJ. Serum lipoprotein (a) levels in patients with first unprovoked venous thromboembolism is not associated with subsequent risk of recurrent VTE. Thromb Res 2010; 126: 222-6.
19. Memon AA, Sundquist J, Zöller B, Wang X, Dahlbäck B, Svensson PJ, Sundquist K. Apolipoprotein M and the risk of unprovoked recurrent venous thromboembolism. Thromb Res 2014; 133: 322-6.
20. van Stralen KJ, Rosendaal FR, Doggen CJ. Minor injuries as a risk factor for venous thrombosis. Arch Intern Med 2008; 168: 21-6.
21. Timp JF, Lijfering WM, Flinterman LE, van Hylckama Vlieg A, le Cessie S, Rosendaal FR, Cannegieter SC. Predictive value of factor VIII levels for recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2015; 13: 1823-32.
22. Flinterman LE, van Hylckama Vlieg A, Cannegieter SC, Rosendaal FR. Long-term survival in a large cohort of patients with venous thrombosis: incidence and predictors. PLoS Med 2012; 9: e1001155.
23. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18: 499-502.
24. Morelli VM, Lijfering WM, Rosendaal FR, Cannegieter SC. Lipid levels and risk of venous thrombosis: results from the MEGA-study [Abstract]. J Thromb Haemost 2015; 13: 152.
25. Kyrle PA, Rosendaal FR, Eichinger S. Risk assessment for recurrent venous thrombosis. Lancet 2010; 376: 2032-9.
26. Esteve E, Ricart W, Fernández-Real JM. Dyslipidemia and inflammation: an evolutionary conserved mechanism. Clin Nutr 2005; 24: 16-31.

27. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res 2004; 45: 1169-96.

28. O'Donnell J, Mumford AD, Manning RA, Laffan M. Elevation of FVIII: C in venous thromboembolism is persistent and independent of the acute phase response. Thromb Haemost 2000; 83: 10-3.

29. Tichelaar V, Mulder A, Kluin-Nelemans H, Meijer K. The acute phase reaction explains only a part of initially elevated factor VIII:C levels: a prospective cohort study in patients with venous thrombosis. Thromb Res 2012; 129: 183-6.

Chapter 4

Role of routine laboratory tests in assessing risk of recurrent venous thrombosis: results from the MEGA follow-up study

V.M. Morelli, W.M. Lijfering, S.M. Rezende, A. van Hylckama Vlieg, F.R. Rosendaal, S.C. Cannegieter

To be submitted

ABSTRACT

Background

Kidney function, measured as estimated glomerular filtration rate (eGFR), glucose levels, and hematologic variables (blood cell count) are easily obtainable tests, and have been associated with increased risk of a first venous thrombosis (VT). Whether these routine tests can identify patients at risk for recurrence is unclear.

Objective

To investigate the predictive value of glucose, eGFR and hematologic variables in patients at risk of recurrent VT.

Patients/Methods

Patients with a first VT were followed from discontinuation of anticoagulant treatment. Several percentile categories of eGFR, glucose levels, and hematologic variables were established. Crude incidence rates with 95% confidence intervals (CIs) of recurrence were estimated in each percentile category. Cox regression models were used to compare groups, adjusted for age and sex.

Results

Of 2106 patients followed for a median of 6.9 years, 326 developed recurrence (incidence rate, 2.7/100 patient-years; 95%CI 2.5-3.1). The adjusted hazard ratio for recurrence was 1.5 (95%CI 0.9-2.4) in the lowest eGFR percentile category (<59mL/min/1.73m^2) vs. the reference (≥86mL/min/1.73m^2). Stratification by unprovoked or provoked first events yielded similar results. Effect modification seemed to be present when patients with first unprovoked VT and renal dysfunction were compared with those with first provoked VT and normal kidney function (hazard ratio for recurrence 3.1, 95%CI 1.6-5.9). Glucose levels and hematologic variables were not associated with an increased risk of recurrence.

Conclusions

Testing glucose levels and hematologic variables did not identify patients at increased risk of recurrent VT, and an association between renal dysfunction and recurrence appeared to be slight at most.

INTRODUCTION

Venous thrombosis (i.e., deep vein thrombosis or pulmonary embolism) is a common multicausal disease associated with considerable mortality and morbidity [1], including recurrence [2-4]. The risk of recurrence is high, with a 5-year cumulative incidence after a first event that varies among studies from 12% to 30% [2-4]. Currently, recommendations on duration of anticoagulant treatment are based on the presence of transient provoking risk factors, such as surgery, immobilization or use of oral contraceptives, and active cancer at the time of the first event [5]. However, only about 50% of patients can be classified in the aforementioned categories [6]. This leads to a clinical dilemma in the other 50%, in whom discontinuing anticoagulant treatment may result in a new thrombotic event, while continuing oral anticoagulation is accompanied by an annual major bleeding risk of 1-3% [6-8].

To reduce uncertainty regarding duration of anticoagulant treatment, it is crucial to gain knowledge on good predictors of recurrent venous thrombosis. Some laboratory tests are potentially useful in their ability to predict recurrent venous thrombosis, such as D-dimer [9-10], factor VIII [11-13], and thrombin generation [14-16]. However, these tests have practical drawbacks, like limited availability in clinical laboratories, lack of standardization with respect to methods and reagents in the case of thrombin generation, or influence of anticoagulant treatment on their measurements, including vitamin K antagonists and direct oral anticoagulants [9,10,14-18]. Hence, easily accessible tests, not influenced by anticoagulant treatment, should be evaluated as predictors of recurrent venous thrombosis.

Kidney function, defined according to the estimated glomerular filtration rate (eGFR) [19], glucose levels, and hematologic variables (blood cell count) are often part of routine medical care of thrombosis patients, and have extensively been investigated in relation to the risk of a first event of venous thrombosis. Renal dysfunction, as measured by a decreased eGFR, has consistently been associated with an increased risk of first venous thrombosis, as demonstrated in the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study [20] and other reports, including a meta-analysis of 5 population-based prospective cohort studies [21,22]. High glucose levels, regardless of a known diabetic state, have also been associated with an increased risk of venous thrombosis during the acute setting of the thrombotic event [23,24]. Among the hematologic variables, levels of haematocrit, hemoglobin, and erythrocytes, and particularly high red cell distribution width (RDW) and monocyte count, have been associated with risk of a first venous thrombosis in several studies in the past few years [25-30], including the MEGA study [31]. However, to our knowledge, data on the role of these routine laboratory tests in assessing risk of recurrent venous thrombosis are scarce for hematologic variables [29,32] and eGFR [33], and unknown for glucose levels.

We, therefore, aimed to investigate the predictive value of easily obtainable laboratory tests that are not influenced by anticoagulation on their measurements, including glucose levels, eGFR and hematologic variables, to identify patients at increased risk of recurrent venous thrombosis after discontinuation of anticoagulant treatment. Since patients with a first unprovoked event are at increased risk of recurrence [3,4], subgroup analyses stratified by type of first event were additionally carried out. To address these questions, we used data from the MEGA follow-up study.

METHODS

Patients

Patients were recruited from the MEGA study, details of which have been described elsewhere [34,35]. Briefly, between March 1999 and August 2004, consecutive patients aged 18-70 years with a first objectively confirmed episode of venous thrombosis were included from 6 anticoagulation clinics in the Netherlands. Patients with deep vein thrombosis (DVT) of the leg, pulmonary embolism (PE) or both were included and patients with upper extremity venous thrombosis were excluded from the follow-up study, resulting in 4956 patients (Fig. 1). Of these, 225 did not consent for follow-up, leaving 4731 patients for the MEGA follow-up study. In particular, for the current analyses, patients with active or previous history of malignancy within 5 years before the first event were excluded. In the MEGA study, blood sampling was mainly determined by calendar time, i.e., for logistic reasons patients were asked to provide blood samples up until June 2002 only. As depicted in Fig. 1, among the 4275 consenting patients eligible for follow-up in the present study, 2215 provided blood samples. Lastly, 109 patients were on anticoagulant treatment at the end of follow-up and were excluded, leaving 2106 patients with follow-up starting at the date of discontinuation of anticoagulant treatment. Of note, 64 patients with self-reported diabetes were excluded when assessing the association of glucose levels with recurrence.

Between 2007 and 2009, the vital status of all MEGA participants was acquired from the central Dutch population register [13,36]. Causes of death were obtained from the national register of death certificates at the Central Bureau of Statistics, and encoded according to the *International Classification of Diseases, Tenth Revision, Clinical Modification* (ICD-10- CM). This study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants.

Initial questionnaire (baseline characteristics) and blood sampling

Patients were asked to fill in a standardized questionnaire on many potential risk factors for venous thrombosis [34]. The questionnaire was sent to all patients within

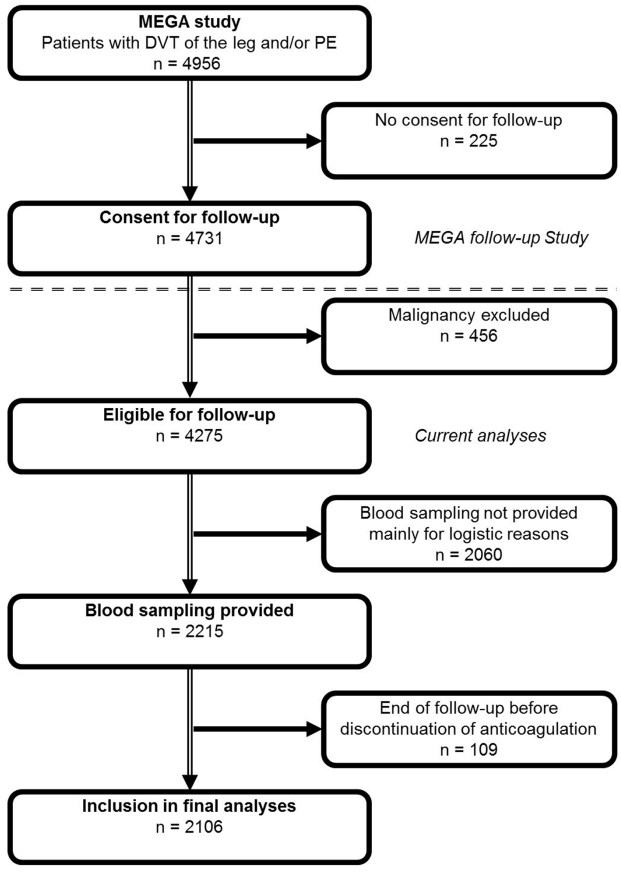

Figure 1. Flowchart of number of the study population. DVT indicates deep vein thrombosis; MEGA, Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study; PE, pulmonary embolism.

a few weeks after registration at the anticoagulation clinic. Of interest for this analysis are the items on body weight and height and self-reported diabetes. Body mass index (BMI) was calculated by dividing weight (in kg) by height squared (m^2). The index date was defined as the date of diagnosis of the first thrombotic event. Unprovoked first venous thrombosis was defined in the absence of trauma, surgery, immobilization (bedridden at home or hospitalization), plaster cast or pregnancy in the first 3 months before the index date, long-distance travel in the first 2 months before the index date, or estrogen use (oral contraceptive or hormonal replacement therapy) at the index date. At least 3 months after discontinuation of anticoagulation, or 1 year after the index date in case of prolonged anticoagulant treatment, patients visited the anticoagulation clinic for an interview and blood sampling for laboratory measurements.

Assessment of recurrent venous thrombosis

Details on the MEGA follow-up study have been previously described [13]. Short questionnaires concerning recurrent venous thrombosis were sent by mail between June 2008 and July 2009 to all consenting patients known to be alive, and a telephone interview was done when questionnaires were not returned. During the same period, information on possible recurrences of all patients was obtained from the anticoagulation clinic where they were initially included for their first event, and in case they moved house, at the clinic near their new address. For all potential recurrences derived from the questionnaire, anticoagulation clinic or both, discharge letters were requested from the clinician who diagnosed the recurrence. Deaths due to recurrent venous thrombosis were counted as fatal recurrent events. On the basis of discharge letters, information from the anticoagulation clinic, short questionnaires filled in by the patients and causes of death, possible recurrences were classified into certain and uncertain events, following a decision rule as previously described [13].

Laboratory measurements

Glucose levels were measured on stored (-80°C) and previously unthawed fasting serum samples by hexokinase method on a Modular P800 Clinical Chemistry analyser (Roche Diagnostics, Mannheim, Germany). eGFR was calculated as previously described in the MEGA study [20] using the Modification of Diet in Renal Disease (MDRD) study equation [19], that takes into account age, sex, ethnicity, and serum creatinine. The hematologic variables i.e., erythrocytes, haematocrit, hemoglobin, monocyte count, and red blood cell indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], and RDW) were assessed as previously described in the MEGA study [31]. All assays were performed by laboratory technicians who were unaware of the status of the samples.

Statistical analysis

Duration of follow-up was defined as the time from the date of discontinuation of anticoagulant treatment to the end of follow-up. The end of follow-up was defined as the date of recurrence, date of death or emigration, or date of filling in the short questionnaire. If patients did not complete the questionnaire, they were censored at the last date we knew them to be recurrence free [13,35] (date of death [n = 17], date of emigration [n = 1], or the date when they were last seen by the anticoagulation clinic or for research purposes [n = 246]). Analyses were limited to certain recurrences (n = 326) and patients with uncertain recurrent events (n = 77) were censored at that time.

Percentile categories of eGFR and glucose levels were defined according to the levels measured in the general population. For this purpose, we used the random digit dialing (RDD) controls from the MEGA study to establish percentile categories for eGFR (≥50th [reference], 10th-50th, 5th-10th, 2.5th-5th and <2.5th percentile) and glucose levels (<50th, 50th-90th [reference], 90th-95th, 95th-97.5th and ≥97.5th percentile). RDD controls with self-reported diabetes were excluded when determining the cutoff points of glucose levels. Fasting glucose levels were also categorized according to the World Health Organization (WHO) clinical cutoff points as <6.1 mmol/L (normal [reference]), 6.1-7.0 mmol/L (impaired fasting glucose), and ≥7.0 mmol/L (diabetes) [37]. Likewise, kidney function was categorized according to the clinical cutoff points of eGFR as ≥90 mL/min per 1.73m^2 (normal kidney function [reference]), 60-90 mL/min per 1.73m^2 (mildly decreased kidney function), and <60 mL/min per 1.73m^2 (moderately to severely decreased kidney function) [20,38]. Hematologic variable categories were defined according to the levels measured in RDD controls from the MEGA study (<10th, 10th-90th [reference], and ≥90th percentile). Given the sex differences in erythrocytes, hematocrit and hemoglobin levels [39], cutoff points were established separately in men and women.

Crude incidence rates with 95% confidence intervals (CIs) of recurrent venous thrombosis were estimated as the number of events over the accumulated follow-up time. Cox proportional hazard regression models were used to obtain hazard ratios (HRs) for recurrence with 95% CIs. Since the aim of this study was to examine the predictive value of eGFR, glucose levels and hematologic variables to identify patients at increased risk of recurrent venous thrombosis, and not to investigate the underlying causal mechanism, we limited adjustments of risk estimates to age and sex, and in the assessment of glucose, for BMI (continuous values) as well. The proportional hazard assumption was verified by evaluating the parallelism between the curves of the log-log survivor function.

In addition to the overall analysis of recurrent venous thrombosis, we performed subgroup analysis stratified by type of first event (provoked or unprovoked events). To quantify potential misclassification of outcomes, several sensitivity analyses were carried out for overall recurrent venous thrombosis: 1) certain and uncertain recurrent events were both taken into account, 2) patients lost to follow-up were all considered to have developed a recurrent event at end of the study (for which the date of the recurrent event was set at the date on which the vital status was checked), and 3) we let follow-up start at the date of blood sampling.

All statistical analyses were performed with SPSS for Windows, release 23.0 (SPSS Inc, Chicago, IL).

RESULTS

Baseline demographic and clinical characteristics

Table 1 describes the baseline characteristics of all patients eligible for follow-up (n = 4275), and those who were included (n = 2106) and excluded (n = 2169) from current analysis. There were no substantial differences in the baseline characteristics of participants who provided blood samples in comparison with those who did not, indicating that the tested patients were representative of the excluded patients eligible for follow-up.

The 2106 patients with a first thrombotic event were followed for a median of 6.9 years (interquartile range [IQR] 2.9-8.0 years). Median age at discontinuation of anticoagulant treatment was 49 years (IQR 38-58 years), and 1161 (55%) patients were women (Table 1). Of the first events, 1464 (70%) were provoked, with trauma, surgery or immobilization accounting for the majority of these events. Most first events were DVTs (n= 1250, 59%). Median time between first event and discontinuation of anticoagulant treatment was 6.0 months (IQR 3.5-6.9 months), and median time between first event and blood collection was 10.0 months (IQR 8.3-12.1 months).

Incidence rates of recurrent venous thrombosis

During a follow-up period of 11,900 patient-years, 326 patients developed recurrent venous thrombosis after discontinuing anticoagulant treatment, yielding an overall incidence rate of 2.7 per 100 patient-years (95% CI 2.5-3.1). As expected, incidence rates were higher for patients with unprovoked first events (4.6 per 100 patient-years, 95% CI 3.9-5.4) compared with those with provoked first events (2.1 per 100 patient-years, 95% CI 1.8-2.4).

Kidney function and risk of recurrent venous thrombosis

Table 2 shows the risk of recurrent venous thrombosis by eGFR percentile categories. Incidence rates of overall recurrence were similar across the higher eGFR percentile categories, ranging from 2.6 to 2.8 per 100 patient-years, and were 4.2 per 100 patient-years (95% CI 2.5-6.5) in the lowest eGFR percentile category, i.e.,<2.5th percentile (<59mL/min/1.73m^2). Compared with patients with an eGFR ≥50th percentile (≥86mL/min/1.73m^2 [reference category]), those with an eGFR <2.5th percentile were 1.5-fold more likely to have experienced a recurrent event of venous thrombosis during the follow-up period (HR 1.5, 95% CI 0.9-2.4 in an age- and sex-adjusted model). Results were virtually the same when clinical cutoff points were used to categorize kidney function instead of percentiles (Table S1). Sensitivity analyses yielded similar results to the main analysis (Table S2).

Table 1. Baseline characteristics

	MEGA follow-up cohort*	Included for analyses	Excluded from analyses
Total	4275	2106	2169
Women, n (%)	2351 (55)	1161 (55)	1190 (55)
Age at discontinuation of anticoagulation, years	49 (38-59)	49 (38-58)	49 (39-60)
Classical venous thrombosis risk factors			
Provoked by, n (%)†	2925 (70)	1464 (70)	1461 (70)
Trauma/surgery/immobilization, n	1661 (57)	824 (56)	837 (57)
Plaster cast, n	214 (7)	108 (7)	106 (7)
Estrogen use (women), n	1322 (45)	696 (48)	626 (43)
Pregnancy/puerperium (women), n	177 (6)	93 (6)	84 (6)
Travel >4hs, n	767 (26)	373 (25)	394 (27)
Unprovoked, n	1252 (30)	612 (30)	640 (30)
Type of index event			
Deep vein thrombosis only, n (%)	2497 (58)	1250 (59)	1247 (57)
Pulmonary embolism ± deep vein thrombosis, n (%)	1778 (42)	856 (41)	922 (43)

Continuous variables are shown as median (25th - 75th percentile), and categorical variables as number (%). Data were missing for some participants in some subgroups.
MEGA, Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis study.
*Patients who consented for follow-up and without active or previous history of malignancy 5 years before the first event.
†As concomitance of provoked risk factors occurred frequently, patients could be counted twice or more.

We next focused on subgroup analysis stratified by type of first event. Relative to the reference, the hazard ratio for recurrent venous thrombosis in patients with a first provoked event was 1.3 (95% CI 0.6-2.9) for the lowest eGFR percentile category (Table 2). Among those with a first unprovoked event, the two lowest eGFR percentile categories were associated with an about 1.5-fold increased risk of recurrent events in comparison with the reference category (Table 2). Similar results were obtained for provoked and unprovoked first events when eGFR clinical cutoff points were used (Table S1). As depicted in Table 3, effect modification seemed to be present when patients with a first unprovoked venous thrombosis and a moderately to severely decreased kidney function (<60mL/min/1.73m^2) were compared with those with a first provoked venous thrombosis and a normal kidney function (≥90mL/min/1.73m^2), with hazard ratio for recurrence of 3.1 (95% CI 1.6-5.9). On an additive scale, this is higher than expected on the basis of the effects of a moderately to severely decreased kidney function and a first unprovoked venous thrombosis separately (1 + 0.4 + 0.7 <3.1).

Table 2. Risk of recurrent venous thrombosis by percentile categories of estimated glomerular filtration rate

eGFR (mL/min/1.73m^2)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Overall analyses (n = 2106)					
≥ 50th (≥86)	1027	5670	151	2.7 (2.3-3.1)	reference
10th-50th (69-86)	774	4519	122	2.7 (2.2-3.2)	1.0 (0.8-1.3)
5th-10th (64-69)	121	738	19	2.6 (1.6-4.0)	0.9 (0.6-1.5)
2.5th-5th (59-64)	87	494	14	2.8 (1.6-4.8)	1.1 (0.6-1.9)
< 2.5th (<59)	97	479	20	4.2 (2.5-6.5)	1.5 (0.9-2.4)
Provoked first event (n = 1464)					
≥ 50th (≥86)	737	4178	89	2.1 (1.7-2.6)	reference
10th-50th (69-86)	545	3246	65	2.0 (1.5-2.6)	1.0 (0.7-1.3)
5th-10th (64-69)	75	476	9	1.9 (0.9-3.6)	0.9 (0.5-1.8)
2.5th-5th (59-64)	54	320	5	1.6 (0.5-3.6)	0.7 (0.3-1.8)
< 2.5th (<59)	53	281	8	2.8 (1.2-5.6)	1.3 (0.6-2.9)
Unprovoked first event (n = 612)					
≥ 50th (≥86)	274	1406	62	4.4 (3.4-5.7)	reference
10th-50th (69-86)	221	1232	56	4.5 (3.4-5.9)	1.0 (0.7-1.5)
5th-10th (64-69)	45	259	10	3.9 (1.9-7.1)	0.9 (0.5-1.8)
2.5th-5th (59-64)	31	166	9	5.4 (2.5-10.3)	1.4 (0.7-2.9)
< 2.5th (<59)	41	183	12	6.6 (3.4-11.5)	1.5 (0.8-2.9)

Estimated glomerular filtration rate percentile categories were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study. The number of patients with provoked and unprovoked events does not add up to a total of 2106 because some data were missing for some variables.
CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age and sex.

Glucose levels and risk of recurrent venous thrombosis

Table 4 shows no association between glucose levels and overall recurrent venous thrombosis, with hazard ratios adjusted for age, sex and BMI ranging from 0.8 (95% CI 0.5-1.4) to 1.0 (95% CI 0.6-1.9) across percentile categories. Similar results were obtained when clinical cutoff points were used (Table S3) and in sensitivity analyses (Table S4). In subgroup analysis stratified by type of first event, we found no consistent association between glucose levels and recurrent venous thrombosis in either provoked or unprovoked first events, irrespective of using percentile categories (Table 4) or clinical cutoff points (Table S3).

Hematologic variables and risk of recurrent venous thrombosis

In Table 5, the risk of recurrent venous thrombosis is presented for percentile categories of erythrocytes, hemoglobin and hematocrit levels according to sex. In

Table 3. Risk of recurrent venous thrombosis by type of first event of venous thrombosis and clinical cutoff points of estimated glomerular filtration rate

Type of first event and eGFR (mL/min/1.73m^2)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Provoked and eGFR ≥ 90	582	3270	71	2.2 (1.7-2.7)	reference
Provoked and eGFR < 60	59	312	8	2.6 (1.1-5.1)	1.4 (0.6-2.9)
Unprovoked and eGFR ≥ 90	206	1107	48	4.3 (3.2-5.7)	1.7 (1.1-2.6)
Unprovoked and eGFR < 60	45	204	14	6.9 (3.8-11.5)	3.1 (1.6-5.9)

eGFR ≥90 mL/min/1.73m^2: normal kidney function; eGFR <60mL/min/1.73m^2: moderately to severely decreased kidney function.
CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age and sex.

Table 4. Risk of recurrent venous thrombosis by percentile categories of glucose levels

Glucose (mmol/L)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Overall analyses (n = 2042)					
< 50th (<4.8)	983	5700	123	2.2 (1.8-2.6)	0.8 (0.6-1.1)
50th-90th (4.8-5.6)	761	4272	137	3.2 (2.7-3.8)	reference
90-95th (5.6-6.0)	143	782	30	3.8 (2.6-5.5)	1.0 (0.7-1.5)
95th-97.5th (6.0-6.6)	88	512	15	2.9 (1.6-4.8)	0.8 (0.5-1.4)
≥ 97.5th (≥ 6.6)	67	338	14	4.1 (2.3-6.9)	1.0 (0.6-1.9)
Provoked first event (n = 1425)					
< 50th (<4.8)	757	4466	74	1.7 (1.3-2.1)	0.9 (0.6-1.2)
50th-90th (4.8-5.6)	494	2881	66	2.3 (1.8-2.9)	reference
90-95th (5.6-6.0)	91	473	19	4.0 (2.4-6.3)	1.4 (0.8-2.4)
95th-97.5th (6.0-6.6)	48	297	9	3.0 (1.4-5.8)	1.3 (0.7-2.6)
≥ 97.5th (≥ 6.6)	35	185	5	2.7 (0.9-6.3)	0.9 (0.3-2.4)
Unprovoked first event (n= 587)					
< 50th (<4.8)	215	1168	49	4.2 (3.1-5.5)	0.8 (0.6-1.2)
50th-90th (4.8-5.6)	252	1321	70	5.3 (4.1-6.7)	reference
90-95th (5.6-6.0)	52	308	11	3.6 (1.8-6.4)	0.7 (0.4-1.3)
95th-97.5th (6.0-6.6)	38	200	6	3.0 (1.1-6.5)	0.6 (0.2-1.3)
≥ 97.5th (≥ 6.6)	30	151	9	6.0 (2.7-11.3)	1.1 (0.5-2.3)

Glucose percentile categories were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.
The number of patients with provoked and unprovoked events does not add up to a total of 2042 because some data were missing for some variables.
CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age, sex and body mass index.

Table 5. Risk of recurrent venous thrombosis by percentile categories of erytrocytes, hemoglobin and hematocrit according to sex

	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Erythrocytes (10^{12}/L)					
Men (n = 932)					
<10th (<4.44)	128	701	25	3.6 (2.3-5.3)	0.9 (0.6-1.3)
10th-90th (4.44-5.28)	676	3613	147	4.1 (3.5-4.8)	reference
≥90th (≥5.28)	128	660	29	4.4 (2.9-6.3)	1.1 (0.7-1.6)
Women (n= 1147)					
<10th (<4.03)	106	594	14	2.4 (1.3-4.0)	1.3 (0.7-2.1)
10th-90th (4.03-4.82)	890	5320	99	1.9 (1.5-2.3)	reference
≥90th (≥4.82)	151	891	8	0.9 (0.4-1.8)	0.5 (0.2-1.0)
Hemoglobin (mmol/L)					
Men (n = 932)					
<10th (<8.47)	103	499	22	4.4 (2.8-6.7)	1.1 (0.7-1.7)
10th-90th (8.47-10.01)	738	4030	159	3.9 (3.4-4.6)	reference
≥90th (≥10.01)	91	445	20	4.5 (2.7-6.9)	1.1 (0.7-1.8)
Women (n= 1147)					
<10th (<7.59)	139	804	13	1.6 (0.9-2.8)	0.8 (0.5-1.4)
10th-90th (7.59-8.91)	845	4964	101	2.0 (1.7-2.5)	reference
≥90th (≥8.91)	163	1037	7	0.7 (0.3-1.4)	0.3 (0.2-0.7)
Hematocrit (L/L)					
Men (n = 932)					
<10th (<0.41)	107	539	20	3.7 (2.3-5.7)	0.9 (0.6-1.5)
10th-90th (0.41-0.47)	720	3921	157	4.00 (3.4-4.7)	reference
≥90th (≥0.47)	105	514	24	4.7 (3.0-6.9)	1.1 (0.7-1.7)
Women (n= 1147)					
<10th (<0.37)	138	759	18	2.4 (1.4-3.7)	1.3 (0.8-2.2)
10th-90th (0.37-0.43)	874	5185	94	1.8 (1.5-2.2)	reference
≥90th (≥0.43)	135	861	9	1.0 (0.5-2.0)	0.6 (0.3-1.2)

Percentile categories of erythrocytes, hemoglobin, and hematocrit were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.
CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age.

men, none of the aforementioned variables were associated with recurrent events, with hazard ratios ranging from 0.9 to 1.1. In women, compared with the reference category, hazard ratios were consistently below 1.0 for levels ≥90th percentile in age adjusted models for erythrocytes (HR 0.5, 95% CI 0.2-1.0), hemoglobin (HR 0.3, 95% CI 0.2-0.7), and hematocrit (HR 0.6, 95% CI 0.3-1.2). Sensitivity analyses yielded similar results for men and women (Tables S5-S7).

Red blood cell indices were not related to recurrent venous thrombosis in overall (Table 6) and subgroup (Table 7) analyses, with the possible exception of MCHC, which was marginally associated with recurrence, particularly for levels above the 90th percentile. Similar results were obtained for overall recurrent events in sensitivity analyses for all red blood cell indices (Tables S8-S10). Monocyte count was not associated with overall recurrent venous thrombosis in main analysis (Table 6) and sensitivity analyses (Tables S8-S10). However, in patients with unprovoked first events, monocyte count below the 10th percentile was associated with a 2.8-fold (95% 1.4-5.8) increased risk of recurrence compared with the reference category (Table 7).

Table 6. Risk of recurrent venous thrombosis by percentile categories of red blood cell indices and monocyte count

	Patients *N*	**Patient-years**	**Recurrent events**	**IR (95% CI)**	**HR* (95% CI)**
Mean corpuscular volume (fL) (n = 2081)					
< 10th (<85.30)	304	1622	40	2.5 (1.8-3.4)	0.9 (0.7-1.3)
10th-90th (85.30-95.70)	1546	8800	246	2.8 (2.5-3.2)	reference
≥90th (≥95.70)	231	1359	37	2.7 (1.9-3.8)	0.9 (0.6-1.2)
Mean corpuscular hemoglobin (fmol) (n = 2081)					
< 10th (<1.76)	269	1473	32	2.2. (1.5-3.1)	0.9 (0.6-1.3)
10th-90th (1.76-2.00)	1545	8869	242	2.7 (2.4-3.1)	reference
≥90th (≥2.00)	267	1439	49	3.4 (2.5-4.5)	1.1 (0.8-1.5)
Mean corpuscular hemoglobin concentration (mmol/L) (n = 2081)					
< 10th (<20.10)	137	799	18	2.3 (1.3-3.6)	1.0 (0.6-1.6)
10th-90th (20.10-21.50)	1778	10216	272	2.7 (2.4-3.0)	reference
≥90th (≥21.50)	166	766	33	4.3 (3.0-6.1)	1.4 (1.0-2.1)
Red cell distribution width (%) (n = 2080)					
< 10th (<11.80)	92	505	13	2.6 (1.4-4.4)	1.0 (0.6-1.7)
10th-90th (11.80-13.70)	1491	8615	235	2.7 (2.4-3.1)	reference
≥90th (≥13.70)	497	2652	75	2.8 (2.2-3.5)	1.1 (0.8-1.4)
Monocytes (x10^9/L) (n = 2057)					
< 10th (<0.22)	77	391	11	2.8 (1.4-5.0)	1.2 (0.7-2.1)
10th-90th (0.22-0.55)	1569	8875	244	2.7 (2.4-3.1)	reference
≥90th (≥0.55)	411	2370	65	2.7 (2.1-3.5)	0.9 (0.7-1.2)

Percentile categories of red blood cell indices and monocyte count were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.

CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age and sex.

Table 7. Risk of recurrent venous thrombosis by percentile categories of red blood cell indices and monocyte count according to type of first event

	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Provoked first event					
Mean corpuscular volume (fL) (n = 1443)					
< 10th (<85.30)	222	1207	21	1.7 (1.1-2.7)	0.8 (0.5-1.3)
10th-90th (85.30-95.70)	1090	6366	137	2.2 (1.8-2.5)	reference
≥90th (≥95.70)	131	815	17	2.1 (1.2-3.3)	0.9 (0.5-1.5)
Mean corpuscular hemoglobin (fmol) (n = 1443)					
< 10th (<1.76)	205	1158	15	1.3 (0.7-2.1)	0.6 (0.4-1.1)
10th-90th (1.76-2.00)	1086	6355	139	2.2 (1.8-2.6)	reference
≥90th (≥2.00)	152	875	21	2.4 (1.5-3.7)	1.0 (0.6-1.6)
Mean corpuscular hemoglobin concentration (mmol/L) (n = 1443)					
< 10th (<20.10)	108	653	9	1.4 (0.6-2.6)	0.8 (0.4-1.5)
10th-90th (20.10-21.50)	1224	7202	149	2.1 (1.8-2.5)	reference
≥90th (≥21.50)	111	533	17	3.2 (1.9-5.1)	1.4 (0.8-2.2)
Red cell distribution width (%) (n = 1442)					
< 10th (<11.80)	69	396	9	2.3 (1.0-4.3)	1.0 (0.5-2.1)
10th-90th (11.80-13.70)	1032	6100	131	2.1 (1.8-2.5)	reference
≥90th (≥13.70)	341	1883	35	1.9 (1.3-2.6)	0.9 (0.6-1.3)
Monocytes (x10^9/L) (n = 1424)					
< 10th (<0.22)	61	319	3	0.9 (0.2-2.7)	0.5 (0.2-1.6)
10th-90th (0.22-0.55)	1091	6324	136	2.2 (1.8-2.5)	reference
≥90th (≥0.55)	272	1621	33	2.0 (1.4-2.9)	0.8 (0.6-1.2)
Unprovoked first event					
Mean corpuscular volume (fL) (n = 608)					
< 10th (<85.30)	78	410	19	4.6 (2.8-7.2)	1.0 (0.6-1.7)
10th-90th (85.30-95.70)	435	2312	109	4.7 (3.9-5.7)	reference
≥90th (≥95.70)	95	517	19	3.7 (2.2-5.7)	0.8 (0.5-1.3)
Mean corpuscular hemoglobin (fmol) (n = 608)					
< 10th (<1.76)	62	310	17	5.4 (3.2-8.8)	1.3 (0.8-2.3)
10th-90th (1.76-2.00)	438	2398	103	4.3 (3.5-5.2)	reference
≥90th (≥2.00)	108	531	27	5.1 (3.4-7.4)	1.2 (0.8-1.8)
Mean corpuscular hemoglobin concentration (mmol/L) (n = 608)					
< 10th (<20.10)	29	147	9	6.1 (2.8-11.6)	1.5 (0.8-3.1)
10th-90th (20.10-21.50)	527	2874	122	4.2 (3.5-5.1)	reference
≥90th (≥21.50)	52	218	16	7.3 (4.2-11.9)	1.6 (1.0-2.7)
Red cell distribution width (%) (n = 608)					
< 10th (<11.80)	21	93	4	4.3 (1.2-11.0)	0.9 (0.4-2.6)
10th-90th (11.80-13.70)	438	2403	103	4.3 (3.5-5.2)	reference
≥90th (≥13.70)	149	743	40	5.4 (3.8-7.3)	1.3 (0.9-1.9)

Table 7. (continued)

	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Monocytes (x10^9/L) (n = 603)					
< 10th (<0.22)	13	59	8	13.6 (5.9-26.7)	2.8 (1.4-5.8)
10th-90th (0.22-0.55)	457	2436	108	4.4 (3.6-5.4)	reference
≥90th (≥0.55)	133	721	31	4.3 (2.9-6.1)	1.0 (0.6-1.4)

Percentile categories of red blood cell indices and monocyte count were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study. The number of patients with provoked and unprovoked events does not add up the total number of patients because some data were missing for some variables. CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age and sex.

DISCUSSION

In this large cohort study of patients with a first venous thrombosis, followed for a prolonged time after stopping anticoagulant treatment, we evaluated the value of inexpensive and easily obtainable tests, not influenced by anticoagulation, to identify patients at increased risk of recurrent venous thrombosis. Overall, renal dysfunction appeared to be associated with a slight increase in the risk of recurrent venous thrombosis, whereas no consistent associations were observed for glucose levels, red blood cell indices and monocyte count. In women only, levels of hemoglobin and the related hematologic variables, i.e., erythrocytes and hematocrit, exceeding the 90th percentile of the distribution of the general population appeared to be inversely associated with risk of recurrent venous thrombosis.

Only a few cohort studies have addressed the role of renal dysfunction in the risk of recurrent venous thrombosis [33,40]. In the Worcester Venous Thromboembolism study [33], a population-based study composed of 1509 venous thrombosis patients, an eGFR <30ml/min/1.73m^2 was associated with 1.57-fold (95% CI 0.95-2.59) increased risk of recurrence in crude analyses compared with a normal kidney function (eGFR ≥90ml/min/1.73m^2) during a 3-year follow-up. Subgroup analyses stratified by provoked or unprovoked first events have not been performed in the above mentioned study. In the Prevention of Renal and Vascular End Stage Disease (PREVEND) study [40], a population-based cohort study, albuminuria, instead of eGFR, was used as a measure of renal dysfunction. Of 351 patients with a first venous thrombosis followed for a median of 3.3 years, elevated albuminuria, defined as levels ≥20 mg/L, was associated with an increased risk of recurrence compared with normal levels (<20mg/L) but mostly in patients with a first unprovoked event. In our study, an eGFR below the 2.5th percentile of the distribution of the general population was associated

with a slight increase in the risk of recurrence in patients with either a first provoked (HR 1.3) or unprovoked (HR 1.5) event. Our results seem to confirm the Worcester and PREVEND studies, even though they should be handled with caution as confidence intervals were wide and included 1. It is of interest that compared with a population at a low baseline risk of recurrence (i.e., patients with a first provoked event and a normal kidney function), we found that having both an unprovoked event and moderately to severely decreased kidney function resulted in a synergic effect on the outcome, with a 3.1-fold increased risk of recurrent venous thrombosis. Taken together, on the basis of our and other studies [33,40], renal dysfunction appears to predict recurrence to some extent. Still, given the increased risk of major bleeding in patients with renal dysfunction during anticoagulant treatment [5,33,41], studies with larger samples sizes, using a uniform measurement of kidney function, are needed to allow a detailed assessment of the ability of a decreased eGFR to identify patients at increased risk of recurrent venous thrombosis, in particular, those with unprovoked first events.

To our knowledge, this is the first follow-up study investigating the predictive value of glucose levels to identify patients at increased risk of recurrent venous thrombosis. We found no consistent association between glucose levels and risk of recurrence, across percentile categories or clinical cutoff points, in overall or subgroup analyses stratified by type of first event. Interestingly, in a case-control study in which glucose levels were measured shortly after a first thrombotic event, the association between glucose levels and venous thrombosis seemed to be explained, at least in part, by the acute phase reaction, as risk estimates declined considerably upon adjustment for C-reactive protein [24]. In follow-up studies, no association was observed between glucose levels, as measured by hemoglobin A_{1c}, and risk of a first venous thrombosis [42,43], except among those with a history of diabetes, in whom a mild association seemed present [42]. On the whole, according to our findings, glucose levels seem unsuitable to predict recurrent events.

Regarding the hematologic variables, few studies have investigated their impact on the risk of recurrent venous thrombosis. For instance, in the Tromsø Study, a population-based cohort study, Ellingsen *et al*. found that high RDW levels predict all-cause mortality in venous thrombosis patients but not recurrent events [29]. Likewise, we found that RDW levels did not identify patients at increased risk of recurrence, including those with unprovoked first events. Even though there is evidence of an association between high RDW levels and an increased risk of a first venous thrombosis, as demonstrated by the MEGA study [31], and later confirmed by other reports [28-30], one should consider that risk factors for a first event do not necessarily predict recurrence [44]. For instance, age, a strong risk factor for a first venous thrombosis, has no effect on recurrence risk [45]. Inherited thrombophilia,

a classic risk factor for a first event, only poorly predicts recurrence in unselected patients [3].

In specific groups, a monocyte count below the 10th percentile of the distribution of the general population was associated with an increased risk of recurrence in patients with unprovoked first events, and levels of hemoglobin, erythrocytes and hematocrit exceeding the 90th percentile were inversely related to recurrence risk in women. It is important to address that in these subgroup analyses, the number of events in the upper (for hemoglobin and related variables) and lower (for monocytes) percentile categories was low, and our results should, therefore, be interpreted with caution. Notably, in the Austrian Study on Recurrent Venous Thromboembolism, composed of 653 patients with a first unprovoked venous thrombosis followed for a mean of 43 months, a high hematocrit was associated with increased risk of recurrence in women only [32]. The source and selection criteria of the study population, the duration of follow-up, the definition of cutoff points, and the limited statistical power of the individual studies might have accounted for the differences between our and the Austrian cohort on the association of haematocrit levels with recurrence.

The strengths of this study include that this is the largest population-based cohort study that has been performed on this issue, in which several laboratory tests used in routine care of thrombosis patients were investigated in their ability to identify patients at increased risk of recurrent venous thrombosis. Patients were followed for a long period of time for a recurrent event that was objectively confirmed and strictly classified as such [13]. Furthermore, because of the large sample size, subgroup analyses stratified by type of first event and several sensitivity analyses could be performed.

Limitations of our study should also be pointed out. First, because of low numbers, we were unable to assess the effect of eGFR, glucose levels and hematologic variables on the risk of recurrence within the more extreme percentile categories. Values in the very low or high percentile categories of these tests could still be clinically relevant, and identify a subset of patients at increased risk of recurrent venous thrombosis. Likewise, because of low numbers due to stratification by sex, we could not determine the effect of hemoglobin, erythrocytes and hematocrit on the risk of recurrence in patients with provoked or unprovoked first events. Second, owing to the design of cohort studies, there was a time lag between the exposure (assessment of routine laboratory tests) and outcome (recurrence). Values of routine laboratory tests might have changed over time in both groups, with and without recurrence, because of prescription of glucose-lowering drugs, changes in lifestyle (i.e. diet and physical activity), aging, or development or progression of comorbidities, which could have resulted in an underestimation of the effect of routine laboratory test values on

the risk of recurrence. Third, patients were included in this study if they experienced their first event of venous thrombosis before the age of 70 years, and our results may therefore not be generalizable to an elderly population (i.e. > 70 years old). Fourth, as blood was collected after the first event of venous thrombosis, values of some of the laboratory tests might have been affected by acute-phase reactions brought about by the thrombotic event, such as glucose levels [46] and monocyte count [47]. However, to avoid this problem blood was drawn at a median of 10 months after the thrombotic event, by which time the effects of the acute-phase reaction would have worn off [48,49].

In summary, testing glucose levels and hematologic variables did not identify patients at increased risk of recurrent venous thrombosis, including those with unprovoked first events, and an association between renal dysfunction and recurrence appeared to be slight at most. Based on our findings, these routine laboratory tests should not guide decisions to extend anticoagulant treatment.

REFERENCES

1. Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 2007; 5: 692-9.
2. Hansson PO, Sörbo J, Eriksson H. Recurrent venous thromboembolism after deep vein thrombosis: incidence and risk factors. Arch Intern Med 2000; 160: 769-74.
3. Christiansen SC, Cannegieter SC, Koster T, Vandenbroucke JP, Rosendaal FR. Thrombophilia, clinical factors, and recurrent venous thrombotic events. JAMA 2005; 293: 2352-61.
4. Prandoni P, Noventa F, Ghirarduzzi A, Pengo V, Bernardi E, Pesavento R, Iotti M, Tormene D, Simioni P, Pagnan A. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. Haematologica 2007; 92: 199-205.
5. Kearon C, Akl EA, Ornelas J, Blaivas A, Jimenez D, Bounameaux H, Huisman M, King CS, Morris TA, Sood N, Stevens SM, Vintch JR, Wells P, Woller SC, Moores L. Antithrombotic Therapy for VTE Disease: CHEST Guideline and Expert Panel Report. Chest 2016; 149: 315-52.
6. Lijfering WM, Biedermann JS, Kruip MJ, Leebeek FW, Rosendaal FR, Cannegieter SC. Can we prevent venous thrombosis with statins: an epidemiologic review into mechanism and clinical utility. Expert Rev Hematol 2016; 9: 1023-30.
7. Kearon C, Gent M, Hirsh J, Weitz J, Kovacs MJ, Anderson DR, Turpie AG, Green D, Ginsberg JS, Wells P, MacKinnon B, Julian JA. A comparison of three months of anticoagulation with extended anticoagulation for a first episode of idiopathic venous thromboembolism. N Engl J Med 1999; 340: 901-7.
8. Veeger NJ, Piersma-Wichers M, Tijssen JG, Hillege HL, Van Der Meer J. Individual time within target range in patients treated with vitamin K antagonists: main determinant of quality of anticoagulation and predictor of clinical outcome. A retrospective study of 2300 consecutive patients with venous thromboembolism. Br J Haematol 2005; 128: 513-9.
9. Verhovsek M, Douketis JD, Yi Q, Shrivastava S, Tait RC, Baglin T, Poli D, Lim W. Systematic review: D-dimer to predict recurrent disease after stopping anticoagulant therapy for unprovoked venous thromboembolism. Ann Intern Med 2008; 149: 481-90.
10. Bruinstroop E, Klok FA, Van De Ree MA, Oosterwijk FL, Huisman MV. Elevated D-dimer levels predict recurrence in patients with idiopathic venous thromboembolism: a meta-analysis. J Thromb Haemost 2009; 7: 611-8.
11. Kyrle PA, Minar E, Hirschl M, Bialonczyk C, Stain M, Schneider B, Weltermann A, Speiser W, Lechner K, Eichinger S. High plasma levels of factor VIII and the risk of recurrent venous thromboembolism. N Engl J Med 2000; 343: 457-62.
12. Legnani C, Benilde C, Michela C, Mirella F, Giuliana G, Gualtiero P. High plasma levels of factor VIII and risk of recurrence of venous thromboembolism. Br J Haematol 2004; 124: 504-510.
13. Timp JF, Lijfering WM, Flinterman LE, van Hylckama Vlieg A, le Cessie S, Rosendaal FR, Cannegieter SC. Predictive value of factor VIII levels for recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2015; 13: 1823-32.

14. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle PA. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. JAMA 2006; 296: 397-402.

15. Eichinger S, Hron G, Kollars M, Kyrle PA. Prediction of recurrent venous thromboembolism by endogenous thrombin potential and D-dimer. Clin Chem 2008; 54: 2042-8.

16. Besser M, Baglin C, Luddington R, van Hylckama Vlieg A, Baglin T. High rate of unprovoked recurrent venous thrombosis is associated with high thrombin-generating potential in a prospective cohort study. J Thromb Haemost 2008; 6: 1720-5.

17. Tichelaar V, de Jong H, Nijland H, Kluin-Nelemans H, Meijer K, Mulder A. Interference of rivaroxaban in one-stage and chromogenic factor VIII:C assays. Thromb Haemost 2011; 106: 990-2.

18. Adcock DM, Gosselin R, Kitchen S, Dwyre DM. The effect of dabigatran on select specialty coagulation assays. Am J Clin Patho 2013; 139: 102-9.

19. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F; Chronic Kidney Disease Epidemiology Collaboration. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. Ann Intern Med 2006; 145: 247-54.

20. Ocak G, Vossen CY, Lijfering WM, Verduijn M, Dekker FW, Rosendaal FR, Cannegieter SC. Role of hemostatic factors on the risk of venous thrombosis in people with impaired kidney function. Circulation 2014; 129: 683-91.

21. Mahmoodi BK, Gansevoort RT, Næss IA, Lutsey PL, Brækkan SK, Veeger NJ, Brodin EE, Meijer K, Sang Y, Matsushita K, Hallan SI, Hammerstrøm J, Cannegieter SC, Astor BC, Coresh J, Folsom AR, Hansen JB, Cushman M. Association of mild to moderate chronic kidney disease with venous thromboembolism: pooled analysis of five prospective general population cohorts. Circulation 2012; 126: 1964-71.

22. Cheung KL, Zakai NA, Folsom AR, Kurella Tamura M, Peralta CA, Judd SE, Callas PW, Cushman M. Measures of Kidney Disease and the Risk of Venous Thromboembolism in the REGARDS (Reasons for Geographic and Racial Differences in Stroke) Study. Am J Kidney Dis 2017; 70: 182-190.

23. Hermanides J, Cohn DM, Devries JH, Kamphuisen PW, Huijgen R, Meijers JC, Hoekstra JB, Büller HR. Venous thrombosis is associated with hyperglycemia at diagnosis: a case-control study. J Thromb Haemost 2009; 7: 945-9.

24. Tichelaar YIGV, Lijfering WM, ter Maaten JC, Kluin-Nelemans JC, Meijer K. High levels of glucose at time of diagnosing venous thrombosis: a case-control study. J Thromb Haemost 2011; 9: 883-5.

25. Braekkan SK, Mathiesen EB, Njølstad I, Wilsgaard T, Hansen JB. Hematocrit and risk of venous thromboembolism in a general population. The Tromso study. Haematologica 2010; 95: 270-5.

26. Basavaraj MG, Brækkan SK, Brodin E, Østerud B, Hansen JB. Monocyte count and procoagulant functions are associated with risk of venous thromboembolism: the Tromsø study. J Thromb Haemost 2011; 9: 1673-6.

27. Cay N, Unal O, Kartal MG, Ozdemir M, Tola M. Increased level of red blood cell distribution width is associated with deep venous thrombosis. Blood Coagul Fibrinolysis 2013; 24: 727-31.

28. Zöller B, Melander O, Svensson P, Engström G. Red cell distribution width and risk for venous thromboembolism: a population-based cohort study. Thromb Res 2014; 133: 334-9.

29. Ellingsen TS, Lappegård J, Skjelbakken T, Brækkan SK, Hansen JB. Red cell distribution width is associated with incident venous thromboembolism (VTE) and case-fatality after VTE in a general population. Thromb Haemost 2015; 113: 193-200.

30. Bucciarelli P, Maino A, Felicetta I, Abbattista M, Passamonti SM, Artoni A, Martinelli I. Association between red cell distribution width and risk of venous thromboembolism. Thromb Res 2015; 136: 590-4.

31. Rezende SM, Lijfering WM, Rosendaal FR, Cannegieter SC. Hematologic variables and venous thrombosis: red cell distribution width and blood monocyte count are associated with an increased risk. Haematologica 2014; 99: 194-200.

32. Eischer L, Tscholl V, Heinze G, Traby L, Kyrle PA, Eichinger S. Hematocrit and the risk of recurrent venous thrombosis: a prospective cohort study. PLoS One 2012; 7: e38705.

33. Parikh AM, Spencer FA, Lessard D, Emery C, Baylin A, Linkletter C, Goldberg RJ. Venous thromboembolism in patients with reduced estimated GFR: a population-based perspective. Am J Kidney Dis 2011; 58: 746-55.

34. van Stralen KJ, Rosendaal FR, Doggen CJ. Minor injuries as a risk factor for venous thrombosis. Arch Intern Med 2008; 168: 21-6.

35. Morelli VM, Lijfering WM, Rosendaal FR, Cannegieter SC. Lipid levels and risk of recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2017; 15: 695-701.

36. Flinterman LE, van Hylckama Vlieg A, Cannegieter SC, Rosendaal FR. Long-term survival in a large cohort of patients with venous thrombosis: incidence and predictors. PLoS Med 2012; 9: e1001155.

37. World Health Organization 2006. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia. http://www.who.int/diabetes/publications/diagnosis_diabetes2006/en/

38. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, Eknoyan G; National Kidney Foundation. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003; 139: 137-47.

39. Murphy WG. The sex difference in haemoglobin levels in adults - mechanisms, causes, and consequences. Blood Rev 2014; 28: 41-7.

40. van Schouwenburg IM, Mahmoodi BK, Veeger NJ, Kluin-Nelemans HC, Gansevoort RT, Meijer K. Elevated albuminuria associated with increased risk of recurrent venous thromboembolism: results of a population-based cohort study. Br J Haematol 2012; 156: 667-71.

41. Kooiman J, den Exter PL, Cannegieter SC, le Cessie S, del Toro J, Sahuquillo JC, Pedrajas JM, Huisman MV. Impact of chronic kidney disease on the risk of clinical outcomes in patients with cancer-associated venous thromboembolism during anticoagulant treatment. J Thromb Haemost 2013; 11: 1968-76.

42. Bell EJ, Selvin E, Lutsey PL, Nambi V, Cushman M, Folsom AR. Glycemia (hemoglobin A1c) and incident venous thromboembolism in the Atherosclerosis Risk in Communities cohort study. Vasc Med 2013; 18: 245-250.

43. Lerstad G, Brodin EE, Enga KF, Jorde R, Schirmer H, Njølstad I, Svartberg J, Braekkan SK, Hansen JB. Hyperglycemia, assessed according to HbA1c, and future risk of venous thromboembolism: the Tromsø study. J Thromb Haemost 2014; 12: 313-9.

44. Cannegieter SC, van Hylckama Vlieg A. Venous thrombosis: understanding the paradoxes of recurrence. J Thromb Haemost 2013; 11 Suppl 1: 161-9.

45. Eischer L, Eichinger S, Kyrle PA. Age at first venous thromboembolism and risk of recurrence: a prospective cohort study. Medicine (Baltimore) 2009; 88: 366-70.

46. Harnish MJ, Lange T, Dimitrov S, Born J, Fehm HL. Differential regulation of human blood glucose level by interleukin-2 and -6. Exp Clin Endocrinol Diabetes 2005; 113: 43-8.

47. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol 2011; 11: 762-74.

48. O'Donnell J, Mumford AD, Manning RA, Laffan M. Elevation of FVIII: C in venous thromboembolism is persistent and independent of the acute phase response. Thromb Haemost 2000; 83: 10-3.

49. Tichelaar V, Mulder A, Kluin-Nelemans H, Meijer K. The acute phase reaction explains only a part of initially elevated factor VIII: C levels: a prospective cohort study in patients with venous thrombosis. Thromb Res 2012; 129: 183-6.

SUPPLEMENTAL MATERIAL

Supplementary Table 1. Risk of recurrent venous thrombosis by clinical cutoff points of estimated glomerular filtration rate

eGFR (mL/min/1.73m^2)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Overall analyses (n = 2106)					
≥90	800	4441	119	2.7 (2.2-3.2)	reference
60-90	1199	6928	185	2.7 (2.3-3.1)	1.0 (0.8-1.2)
<60	107	531	22	4.1 (2.6-6.3)	1.5 (0.9-2.4)
Provoked first event (n = 1464)					
≥90	582	3270	71	2.2 (1.7-2.7)	reference
60-90	823	4919	97	2.0 (1.6-2.4)	0.9 (0.7-1.3)
<60	59	312	8	2.6 (1.1-5.1)	1.2 (0.6-2.6)
Unprovoked first event (n = 612)					
≥90	206	1107	48	4.3 (3.2-5.7)	reference
60-90	361	1935	87	4.5 (3.6-5.5)	1.1 (0.7-1.5)
<60	45	204	14	6.9 (3.8-11.5)	1.6 (0.9-3.1)

eGFR ≥90 mL/min/1.73m^2: normal kidney function; eGFR 60-90 mL/min/1.73m^2: mildly decreased kidney function; eGFR <60mL/min/1.73m^2: moderately to severely decreased kidney function. The number of patients with provoked and unprovoked events does not add up to a total of 2106 because some data were missing for some variables.

CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age and sex.

Supplementary Table 2. Risk of overall recurrent venous thrombosis by percentile categories and clinical cutoff points of estimated glomerular filtration rate

eGFR (mL/min/1.73m^2)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Sensitivity analysis 1 (n = 2106)					
Percentile categories†					
> 50th (≥86)	1027	5670	191	3.4 (2.9-3.9)	reference
10th-50th (69-86)	774	4519	145	3.2 (2.7-3.8)	0.9 (0.7-1.2)
5th-10th (64-69)	121	738	22	3.0 (1.9-4.5)	0.8 (0.5-1.3)
2.5th-5th (59-64)	87	494	19	3.8 (2.3-6.0)	1.1 (0.7-1.9)
< 2.5th (<59)	97	479	26	5.4 (3.6-8.0)	1.5 (1.0-2.3)
Clinical cutoff points‡					
≥90	800	4441	147	3.3 (2.8-3.9)	reference
60-90	1199	6928	228	3.3 (2.9-3.7)	1.0 (0.8-1.2)
<60	107	531	28	5.3 (3.5-7.6)	1.5 (1.0-2.3)
Sensitivity analysis 2 (n = 2106)					
Percentile categories†					
> 50th (≥86)	1027	5670	288	5.1 (4.5-5.7)	reference
10th-50th (69-86)	774	4519	204	4.5 (3.9-5.2)	0.9 (0.8-1.1)
5th-10th (64-69)	121	738	28	3.8 (2.5-5.5)	0.8 (0.5-1.2)
2.5th-5th (59-64)	87	494	21	4.3 (2.6-6.5)	0.9 (0.6-1.4)
< 2.5th (<59)	97	479	32	6.7 (4.6-9.4)	1.3 (0.9-1.9)
Clinical cutoff points‡					
≥90	800	4441	224	5.0 (4.5-5.7)	reference
60-90	1199	6928	314	4.5 (4.0-5.1)	0.9 (0.8-1.1)
<60	107	531	35	6.6 (4.6-9.2)	1.3 (0.9-1.9)
Sensitivity analysis 3 (n = 1915)					
Percentile categories†					
> 50th (≥86)	915	5575	131	2.4 (2.0-2.7)	reference
10th-50th (69-86)	715	4428	110	2.5 (2.0-3.0)	1.0 (0.8-1.3)
5th-10th (64-69)	110	728	13	1.8 (1.0-3.1)	0.7 (0.4-1.3)
2.5th-5th (59-64)	79	487	11	2.3 (1.1-4.0)	1.0 (0.5-1.8)
< 2.5th (<59)	96	527	21	4.0 (2.5-6.1)	1.6 (1.0-2.6)
Clinical cutoff points‡					
≥90	711	4348	103	2.4 (1.9-2.9)	reference
60-90	1098	6808	160	2.4 (2.0-2.7)	1.0 (0.8-1.3)
<60	106	589	23	3.9 (2.5-5.9)	1.6 (1.0-2.6)

CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; IR, incidence rate per 100 patient-years.Sensitivity analysis 1: certain and uncertain recurrent events were taken into account. Sensitivity analysis 2: patients lost to follow-up were all considered to have developed a recurrent event at the end of the study. Sensitivity analysis 3: start of follow-up from blood sampling.

*Adjusted for age and sex. †eGFR percentile categories were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study. ‡eGFR ≥90 mL/min/1.73m^2: normal kidney function; eGFR 60-90 mL/min/1.73m^2: mildly decreased kidney function; eGFR <60mL/min/1.73m^2: moderately to severely decreased kidney function.

Supplementary Table 3. Risk of recurrent venous thrombosis by clinical cutoff points of glucose levels

Glucose (mmol/L)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Overall analyses (n = 2042)					
< 6.1	1913	10892	296	2.7 (2.4-3.1)	reference
6.1-7.0	84	486	16	3.3 (1.9-5.3)	1.0 (0.6-1.6)
≥ 7.0	45	226	7	3.1 (1.2-6.4)	0.8 (0.4-1.8)
Provoked first event (n = 1425)					
< 6.1	1357	7903	162	2.1 (1.7-2.4)	reference
6.1-7.0	44	267	8	3.00 (1.3-5.9)	1.3 (0.7-2.7)
≥ 7.0	24	132	3	2.3 (0.5-6.6)	0.6 (0.2-2.4)
Unprovoked first event (n = 587)					
< 6.1	529	2844	133	4.7 (3.9-5.5)	reference
6.1-7.0	39	212	8	3.8 (1.6-7.4)	0.7 (0.3-1.5)
≥ 7.0	19	92	4	4.3 (1.2-11.1)	1.0 (0.4-2.7)

Glucose levels were categorized according to the clinical cutoff points from the World Health Organization: <6.1 mmol/L, normal fasting glucose; 6.1-7.0 mmol/L, impaired fasting glucose; ≥ 7.0 mmol/L, fasting glucose corresponding to diabetes.

The number of patients with provoked and unprovoked events does not add up to a total of 2042, because some data were missing for some variables.

CI, confidence interval;HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age, sex and body mass index.

Supplementary Table 4. Risk of overall recurrent venous thrombosis by percentile categories and clinical cutoff points of glucose levels

Glucose (mmol/L)	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Sensitivity analysis 1 (n = 2042)					
Percentile categories†					
< 50th (<4.8)	983	5700	158	2.8 (2.4-3.2)	0.9 (0.7-1.2)
50th-90th (4.8-5.6)	761	4272	161	3.8 (3.2-4.4)	reference
90-95th (5.6-6.0)	143	782	33	4.2 (2.9-5.9)	1.0 (0.7-1.4)
95th-97.5th (6.0-6.6)	88	512	21	4.1 (2.5-6.3)	0.9 (0.6-1.5)
≥ 97.5th (≥ 6.6)	67	338	18	5.3 (3.2-8.4)	1.2 (0.7-1.9)
Clinical cutoff points‡					
< 6.1	1913	10892	358	3.3 (3.0-3.6)	reference
6.1-7.0	84	486	24	4.9 (3.1-7.3)	1.1 (0.7-1.8)
≥ 7.0	45	226	9	4.0 (1.8-7.6)	0.9 (0.4-1.8)
Sensitivity analysis 2 (n = 2042)					
Percentile categories†					
< 50th (<4.8)	983	5700	239	4.2 (3.7-4.8)	0.9 (0.7-1.1)
50th-90th (4.8-5.6)	761	4272	219	5.1 (4.5-5.9)	reference
90-95th (5.6-6.0)	143	782	48	6.1 (4.5-8.1)	1.1 (0.8-1.5)
95th-97.5th (6.0-6.6)	88	512	23	4.5 (2.8-6.7)	0.9 (0.6-1.4)
≥ 97.5th (≥ 6.6)	67	338	24	7.1 (4.5-10.6)	1.1. (0.7-1.8)
Clinical cutoff points‡					
< 6.1	1913	10892	516	4.7 (4.3-5.2)	reference
6.1-7.0	84	486	22	4.5 (2.8-6.9)	0.9 (0.6-1.4)
≥ 7.0	45	226	15	6.6 (3.7-10.9)	1.1 (0.6-1.9)
Sensitivity analysis 3 (n = 1861)					
Percentile categories†					
< 50th (<4.8)	886	5558	106	1.9 (1.6-2.3)	0.8 (0.6-1.1)
50th-90th (4.8-5.6)	695	4246	122	2.9 (2.4-3.4)	reference
90-95th (5.6-6.0)	134	787	27	3.4 (2.3-5.0)	1.0 (0.7-1.6)
95th-97.5th (6.0-6.6)	84	499	12	2.4 (1.2-4.2)	0.8 (0.4-1.4)
≥ 97.5th (≥ 6.6)	62	327	14	4.3 (2.3-7.2)	1.2 (0.6-2.1)
Clinical cutoff points‡					
< 6.1	1736	10722	260	2.4 (2.1-2.7)	reference
6.1-7.0	83	470	14	3.0 (1.6-5.0)	1.0 (0.6-1.7)
≥ 7.0	42	225	7	3.1 (1.3-6.4)	0.9 (0.4-2.0)

CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years. Sensitivity analysis 1: certain and uncertain recurrent events were taken into account. Sensitivity analysis 2: patients lost to follow-up were all considered to have developed a recurrent event at the end of the study. Sensitivity analysis 3: start of follow-up from blood sampling. *Adjusted age, sex, and body mass index. †Glucose percentile categories were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study. ‡Glucose levels were categorized according to the clinical cutoff points from the World Health Organization: <6.1 mmol, normal fasting glucose; 6.1-7.0 mmol/L, impaired fasting glucose; ≥ 7.0 mmol/L, fasting glucose corresponding to diabetes.

Supplementary Table 5. Risk of recurrent venous thrombosis by percentile categories of erytrocytes, hemoglobin and hematocrit according to sex when certain and uncertain events were taken into account

	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Erythrocytes (10^{12}/L)					
Men (n = 932)					
<10th (<4.44)	128	701	26	3.7 (2.4-5.4)	0.7 (0.5-1.1)
10th-90th (4.44-5.28)	676	3613	180	5.0 (4.3-5.8)	reference
≥90th (≥5.28)	128	660	38	5.8 (4.1-7.9)	1.2 (0.8-1.6)
Women (n = 1147)					
<10th (<4.03)	106	594	17	2.9 (1.7-4.6)	1.2 (0.7-2.0)
10th-90th (4.03-4.82)	890	5320	124	2.3 (1.9-2.8)	reference
≥90th (≥4.82)	151	891	12	1.3 (0.7-2.4)	0.6 (0.3-1.1)
Hemoglobin (mmol/L)					
Men (n = 932)					
<10th (<8.47)	103	499	23	4.6 (2.9-6.9)	0.9 (0.6-1.4)
10th-90th (8.47-10.01)	738	4030	194	4.8 (4.2 -5.5)	reference
≥90th (≥10.01)	91	445	27	6.1 (4.0-8.8)	1.2 (0.8-1.9)
Women (n = 1147)					
<10th (<7.59)	139	804	15	1.9 (1.0-3.1)	0.7 (0.4-1.3)
10th-90th (7.59-8.91)	845	4964	127	2.6 (2.1-3.0)	reference
≥90th (≥8.91)	163	1037	11	1.1 (0.5-1.9)	0.4 (0.2-0.8)
Hematocrit (L/L)					
Men (n = 932)					
<10th (<0.41)	107	539	22	4.1 (2.6-6.2)	0.8 (0.5-1.3)
10th-90th (0.41-0.47)	720	3921	189	4.8 (4.2-5.6)	reference
≥90th (≥0.47)	105	514	33	6.4 (4.4-9.0)	1.3 (0.9-1.9)
Women (n = 1147)					
<10th (<0.37)	138	759	22	2.9 (1.8-4.4)	1.3 (0.8-2.0)
10th-90th (0.37-0.43)	874	5185	120	2.3 (1.9-2.8)	reference
≥90th (≥0.43)	135	861	11	1.3 (0.6-2.3)	0.6 (0.3-1.0)

Percentile categories of erythrocytes, hemoglobin, and hematocrit were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.

CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age.

Supplementary Table 6. Risk of recurrent venous thrombosis by percentile categories of erytrocytes, hemoglobin and hematocrit according to sex when patients lost to follow-up were all considered to have developed a recurrent event at the end of the study

	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Erythrocytes (10^{12}/L)					
Men (n = 932)					
<10th (<4.44)	128	701	46	6.6 (4.8-8.8)	1.1 (0.8-1.6)
10th-90th (4.44-5.28)	676	3613	216	6.0 (5.2-6.8)	reference
≥90th (≥5.28)	128	660	42	6.4 (4.6-8.6)	1.0 (0.8-1.5)
Women (n = 1147)					
<10th (<4.03)	106	594	28	4.7 (3.1-6.8)	1.2 (0.8-1.8)
10th-90th (4.03-4.82)	890	5320	202	3.8 (3.3-4.4)	reference
≥90th (≥4.82)	151	891	30	3.4 (2.3-4.8)	0.9 (0.6-1.3)
Hemoglobin (mmol/L)					
Men (n = 932)					
<10th (<8.47)	103	499	40	8.0 (5.7-10.9)	1.4 (1.0-1.9)
10th-90th (8.47-10.01)	738	4030	233	5.8 (5.1-6.6)	reference
≥90th (≥10.01)	91	445	31	7.0 (4.7-9.9)	1.2 (0.8-1.7)
Women (n = 1147)					
<10th (<7.59)	139	804	34	4.2 (2.9-5.9)	1.0 (0.7-1.5)
10th-90th (7.59-8.91)	845	4964	203	4.1 (3.5-4.7)	reference
≥90th (≥8.91)	163	1037	23	2.2 (1.4-3.3)	0.6 (0.4-0.9)
Hematocrit (L/L)					
Men (n = 932)					
<10th (<0.41)	107	539	39	7.2 (5.1-9.9)	1.3 (0.9-1.7)
10th-90th (0.41-0.47)	720	3921	229	5.8 (5.1-6.6)	reference
≥90th (≥0.47)	105	514	36	7.0 (4.9-9.7)	1.2 (0.8-1.7)
Women (n = 1147)					
<10th (<0.37)	138	759	38	5.0 (3.5-6.8)	1.3 (0.9-1.8)
10th-90th (0.37-0.43)	874	5185	198	3.8 (3.3-4.4)	reference
≥90th (≥0.43)	135	861	24	2.8 (1.8-4.1)	0.8 (0.5-1.2)

Percentile categories of erythrocytes, hemoglobin, and hematocrit were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.

CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age.

Supplementary Table 7. Risk of recurrent venous thrombosis by percentile categories of erytrocytes, hemoglobin and hematocrit according to sex with start of follow-up from blood sampling

	Patients *N*	Patient-years	Recurrent events	IR (95% CI)	HR* (95% CI)
Erythrocytes (10^{12}/L)					
Men (n = 847)					
<10th (<4.44)	114	673	24	3.6 (2.3-5.3)	1.1 (0.7-1.6)
10th-90th (4.44-5.28)	615	3618	120	3.3 (2.7-4.0)	reference
≥90th (≥5.28)	118	681	25	3.7 (2.4-5.4)	1.1 (0.7-1.7)
Women (n = 1046)					
<10th (<4.03)	97	576	13	2.3 (1.2-3.9)	1.3 (0.7-2.3)
10th-90th (4.03-4.82)	809	5186	91	1.8 (1.4-2.2)	reference
≥90th (≥4.82)	140	892	9	1.0 (0.5-1.9)	0.6 (0.3-1.2)
Hemoglobin (mmol/L)					
Men (n = 847)					
<10th (<8.47)	88	522	17	3.3 (1.9-5,2)	1.0 (0.6-1.6)
10th-90th (8.47-10.01)	678	4009	135	3.4 (2.8-4.0)	reference
≥90th (≥10.01)	81	441	17	3.9 (2.5-6.2)	1.1 (0.7-1.9)
Women (n = 1046)					
<10th (<7.59)	129	797	14	1.8 (1.0-2.9)	0.9 (0.5-1.6)
10th-90th (7.59-8.91)	764	4833	92	1.9 (1.5-2.3)	reference
≥90th (≥8.91)	153	1024	7	0.7 (0.3-1.4)	0.4 (0.2-0.8)
Hematocrit (L/L)					
Men (n = 847)					
<10th (<0.41)	92	547	16	2.9 (1.7-4.8)	0.9 (0.5-1.4)
10th-90th (0.41-0.47)	661	3909	132	3.4 (2.8-4.0)	reference
≥90th (≥0.47)	94	516	21	4.1 (2.5-6.2)	1.2 (0.8-1.9)
Women (n = 1046)					
<10th (<0.37)	128	747	19	2.5 (1.5-4.0)	1.5 (0.9-2.5)
10th-90th (0.37-0.43)	792	5054	85	1.7 (1.3-2.1)	reference
≥90th (≥0.43)	126	853	9	1.1 (0.5-2.0)	0.6 (0.3-1.3)

Percentile categories of erythrocytes, hemoglobin, and hematocrit were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.

CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age.

Supplementary Table 8. Risk of recurrent venous thrombosis by percentile categories of red blood cell indices and monocyte count when certain and uncertain events were taken into account

	Patients *N*	**Patient-years**	**Recurrent events**	**IR (95% CI)**	**HR* (95% CI)**
Mean corpuscular volume (fL) (n = 2081)					
< 10th (<85.30)	304	1622	56	3.5 (2.6-4.5)	1.1 (0.8-1.4)
10th-90th (85.30-95.70)	1546	8800	299	3.4 (3.0-3.8)	reference
≥90th (≥95.70)	231	1359	43	3.1 (2.3-4.3)	0.8 (0.6-1.2)
Mean corpuscular hemoglobin (fmol) (n = 2081)					
< 10th (<1.76)	269	1473	44	3.0 (2.2-4.0)	1.0 (0.8-1.4)
10th-90th (1.76-2.00)	1545	8869	294	3.3 (2.9-3.7)	reference
≥90th (≥2.00)	267	1439	60	4.2 (3.2-5.4)	1.1 (0.8-1.5)
Mean corpuscular hemoglobin concentration (mmol/L) (n = 2081)					
< 10th (<20.10)	137	799	23	2.9 (1.8-4.3)	1.0 (0.7-1.6)
10th-90th (20.10-21.50)	1778	10216	333	3.3 (2.9-3.6)	reference
≥90th (≥21.50)	166	766	42	5.5 (3.9-7.4)	1.5 (1.1-2.1)
Red cell distribution width (%) (n = 2080)					
< 10th (<11.80)	92	505	14	2.8 (1.5-4.7)	0.9 (0.5-1.5)
10th-90th (11.80-13.70)	1491	8615	284	3.3 (2.9-3.7)	reference
≥90th (≥13.70)	497	2652	100	3.8 (3.1-4.6)	1.2 (0.9-1.5)
Monocytes (x10^9/L) (n = 2057)					
< 10th (<0.22)	77	391	12	3.1 (1.6-5.4)	1.1 (0.6-1.9)
10th-90th (0.22-0.55)	1569	8875	295	3.3 (3.0-3.7)	reference
≥90th (≥0.55)	411	2370	87	3.7 (2.9-4.5)	1.0 (0.8-1.3)

Percentile categories of red blood cell indices and monocyte count were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.
CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age and sex.

Supplementary Table 9. Risk of recurrent venous thrombosis by percentile categories of red blood cell indices and monocyte count when patients lost to follow-up were all considered to have developed a recurrent event at end of the study

	Patients *N*	**Patient-years**	**Recurrent events**	**IR (95% CI)**	**HR* (95% CI)**
Mean corpuscular volume (fL) (n = 2081)					
< 10th (<85.30)	304	1622	81	5.0 (4.0-6.2)	1.0 (0.8-1.3)
10th-90th (85.30-95.70)	1546	8800	424	4.8 (4.4-5.3)	reference
≥90th (≥95.70)	231	1359	61	4.5 (3.4-5.8)	0.9 (0.7-1.2)
Mean corpuscular hemoglobin (fmol) (n = 2081)					
< 10th (<1.76)	269	1473	70	4.8 (3.7-6.0)	1.1 (0.8-1.4)
10th-90th (1.76-2.00)	1545	8869	416	4.7 (4.3-5.2)	reference
≥90th (≥2.00)	267	1439	80	5.6 (4.4-6.9)	1.1 (0.9-1.4)
Mean corpuscular hemoglobin concentration (mmol/L) (n = 2081)					
< 10th (<20.10)	137	799	36	4.5 (3.2-6.2)	1.1 (0.8-1.5)
10th-90th (20.10-21.50)	1778	10216	475	4.6 (4.2-5.1)	reference
≥90th (≥21.50)	166	766	55	7.2 (5.4-9.3)	1.3 (1.0-1.8)
Red cell distribution width (%) (n = 2080)					
< 10th (<11.80)	92	505	27	5.3 (3.5-7.8)	1.1 (0.8-1.7)
10th-90th (11.80-13.70)	1491	8615	401	4.7 (4.2-5.1)	reference
≥90th (≥13.70)	497	2652	138	5.2 (4.4-6.1)	1.1 (0.9-1.4)
Monocytes (x10^9/L) (n = 2057)					
< 10th (<0.22)	77	391	24	6.1 (3.9-9.1)	1.3 (0.9-2.0)
10th-90th (0.22-0.55)	1569	8875	432	4.9 (4.4-5.3)	reference
≥90th (≥0.55)	411	2370	106	4.5 (3.7-5.4)	0.9 (0.7-1.1)

Percentile categories of red blood cell indices and monocyte count were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.

CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.

*Adjusted for age and sex.

Supplementary Table 10. Risk of recurrent venous thrombosis by percentile categories of red blood cell indices and monocyte count with start of follow-up from blood sampling

	Patients *N*	**Patient-years**	**Recurrent events**	**IR (95% CI)**	**HR* (95% CI)**
Mean corpuscular volume (fL) (n = 1895)					
< 10th (<85.30)	272	1639	34	2.1 (1.4-2.9)	0.9 (0.6-1.3)
10th-90th (85.30-95.70)	1408	8675	217	2.5 (2.2-2.9)	reference
≥90th (≥95.70)	215	1318	32	2.4 (1.7-3.4)	0.9 (0.6-1.3)
Mean corpuscular hemoglobin (fmol) (n = 1895)					
< 10th (<1.76)	244	1498	30	2.0 (1.4-2.9)	0.9 (0.6-1.4)
10th-90th (1.76-2.00)	1410	8746	211	2.4 (2.1-2.8)	reference
≥90th (≥2.00)	241	1388	42	3.0 (2.2-4.1)	1.1 (0.8-1.6)
Mean corpuscular hemoglobin concentration (mmol/L) (n = 1895)					
< 10th (<20.10)	133	812	18	2.2 (1.3-3.5)	1.1 (0.7-1.8)
10th-90th (20.10-21.50)	1619	10084	239	2.4 (2.1-2.7)	reference
≥90th (≥21.50)	143	736	26	3.5 (2.3-5.2)	1.4 (0.9-2.1)
Red cell distribution width (%) (n = 1894)					
< 10th (<11.80)	83	496	12	2.4 (1.3-4.2)	1.0 (0.6-1.8)
10th-90th (11.80-13.70)	1342	8385	208	2.5 (2.2-2.8)	reference
≥90th (≥13.70)	469	2743	63	2.3 (1.8-2.9)	1.0 (0.7-1.3)
Monocytes (x10^9/L) (n = 1871)					
< 10th (<0.22)	65	379	10	2.6 (1.3-4.9)	1.3 (0.7-2.4)
10th-90th (0.22-0.55)	1426	8784	214	2.4 (2.1-2.8)	reference
≥90th (≥0.55)	380	2330	56	2.4 (1.8-3.1)	0.9 (0.7-1.2)

Percentile categories of red blood cell indices and monocyte count were defined according to the levels measured in random digit dialing controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) Study.
CI, confidence interval; HR, hazard ratio; IR, incidence rate per 100 patient-years.
*Adjusted for age and sex.

Chapter 5

Interrelation between levels of hemostatic factors, lipids and C-reactive protein in population controls

V.M. Morelli, R. Li-Gao, W.M. Lijfering, F.R. Rosendaal,
S.C. Cannegieter, A. van Hylckama Vlieg

To be submitted

ABSTRACT

Background

Previous studies have shown that hemostatic factor levels are interrelated and clustered together. However, results were not consistent, probably due to differences in sample sizes, study population, and variables studied. Furthermore, since arterial and venous thrombosis share some traditional risk factors, we questioned if and how hemostatic factors cluster with lipids and C-reactive protein (CRP).

Objective

To assess the clustering of hemostatic factor levels, and how these clusters relate to lipid and CRP levels.

Methods

We included 2874 individuals (47% men) who had participated as population controls in a previous study. Clusters of interrelated factors were identified by principal component analysis. A factor loading >0.40 was used as the marginal value to include factors in a cluster.

Results

We identified 3 clusters among the hemostatic factors: a vitamin K-dependent factor (VKDF) cluster (factors [F]II, VII, IX, X, protein C and protein S [PS]) that also included FXI and antithrombin (AT); another comprising fibrinogen, FVIII, von Willebrand factor (VWF) and D-dimer, and a third one including FV, tissue factor pathway inhibitor, PS and AT. The addition of lipid fractions and CRP led to two extra lipid clusters, with triglycerides also clustering with VKDFs. VWF and FVIII now formed a separate cluster, and CRP clustered with fibrinogen, D-dimer and FIX. When individuals with malignancies or self-reported chronic diseases were excluded (n=570), the clustering pattern remained virtually the same.

Conclusions

In this comprehensive study, we confirmed and extended clustering patterns of previous reports between levels of hemostatic factors, lipids and CRP.

INTRODUCTION

Previous studies have shown that hemostatic factor levels are interrelated and clustered together [1-3], suggesting that there are common mechanisms located outside the hemostatic factor genes that could regulate levels of several proteins in the hemostatic system [2]. In these studies, levels of procoagulant vitamin K-dependent factors (VKDFs), i.e. factors(F) II, VII, IX and X, consistently clustered together [1-3]. However, the clustering pattern of other hemostatic factors substantially differed among reports, probably due to distinct samples sizes, study population, and hemostatic factors studied [1-3].

It is noteworthy that some hemostatic factors have been shown to cluster with cardiometabolic risk factors. For instance, procoagulant VKDF levels clustered together with lipids, in particular, triglyceride levels [1,4], whereas levels of fibrinogen, FVIII, FIX, and D-dimer clustered with levels of C-reactive protein (CRP) [1], an inflammatory risk marker of arterial cardiovascular disease (CVD) [5]. However, the interrelation between hemostatic factor, lipid and CRP levels is not known in detail, as studies on this topic are few, involve small sample sizes, and study a limited number of lipids and hemostatic factors [1,4]. Clarification of this issue is relevant since several studies in the past decade have shown that venous thrombosis is associated with an increased risk of subsequent arterial CVD [6-10], and that the two diseases may share common risk factors [8,11]. Indeed, cardiometabolic risk factors, such as dyslipidemia, inflammation and obesity, are well known to increase the risk of arterial CVD [12-14] but are also associated with venous thrombosis [15-18]. Knowledge on the interrelation between levels of hemostatic factors, lipids and inflammation could provide insights on mechanisms underlying the associations of cardiometabolic risk factors with venous thrombosis. In addition, these insights may further reveal why lipid-lowering drugs (statins) are not only able to decrease the risk of arterial CVD, but also of venous thrombosis [19].

The aim of this study was to investigate the clustering of hemostatic factor levels, and how these clusters relate to lipid and CRP levels in population controls. For this purpose, we used data from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study.

METHODS

Study population

The study population was comprised of the control group from the MEGA study, details of which have been previously described [20]. Briefly, between March 1999 and September 2004, 4956 consecutive patients aged 18-70 years with a first objectively confirmed deep vein thrombosis of the leg or pulmonary embolism were enrolled

from six anticoagulation clinics in the Netherlands. During the same time period, 6297 control subjects without a history of venous thrombosis were included. Control subjects were either partners of the patients (n = 3297) or individuals approached by random digit dialing [RDD] (n = 3000). In the MEGA study, blood sampling was determined by calendar time, i.e., for logistic reasons participants were asked to provide blood samples up to June 2002 only. Of the 6297 control subjects, 2943 provided blood samples. We excluded 41 individuals because the quality of their plasma was not adequate for blood coagulation assays, leaving 2902 control subjects. For the current analysis, to eliminate any influence of anticoagulant treatment on the interpretation of the results of the hemostatic factors, control subjects on anticoagulation at blood sampling were excluded (n = 28). Therefore, the present study included 2874 population controls, of whom 570 had a history of malignancy or chronic disease, defined as self-reported liver disease, kidney disease, rheumatoid arthritis, multiple sclerosis, diabetes mellitus, hypothyroidism, hyperthyroidism, chronic bronchitis, emphysema, thrombophlebitis, heart failure, hemorrhagic stroke, and arterial CVD (angina, myocardial infarction, peripheral vascular disease, ischemic stroke, and transient ischemic attack). This study was approved by the Ethics Committee of the Leiden University Medical Center, and written informed consent was obtained from all participants.

Laboratory measurements

Blood collection and laboratory measurements have been previously described in the MEGA study [21-24]. Briefly, fibrinogen activity was measured according to the method of Clauss on a STA-R analyzer [25]. FII activity, FVII activity, FVIII activity, FX activity, and FXI activity were measured with a mechanical clot detection method on a STA-R analyzer (Diagnostica Stago) [21]. FV and FIX antigen levels were determined by enzyme-linked immunosorbent assay (ELISA), and von Willebrand factor (VWF) antigen was measured by an immunoturbidimetric method using STA Liatest kit (Diagnostica Stago) [21]. Measurements of antithrombin and protein C levels were performed with a chromogenic assay, whereas total protein S levels were determined by ELISA (Diagnostica Stago) [21]. Total tissue factor pathway inhibitor (TFPI) activity levels were assessed in citrated plasma by measuring TFPI inhibition of the catalytic tissue factor (TF)-factor VIIa (FVIIa) complex using the Actichrome TFPI activity assay (Sekisui Diagnostics); one unit of TFPI activity corresponds to 55 ng/ml plasma TFPI. D-dimer was measured with the D-dimer HemosIL assay on an ACL TOP 700 analyzer (Instrumentation Laboratory) [22]. CRP levels and lipid levels, i.e. triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (apo A1), and apolipoprotein (apo B) were measured as previously described [23,24].

Statistical analysis

Principal component analysis with orthogonal varimax rotation was used to assess the interrelations between levels of hemostatic factors, lipids and CRP, as previously described [2]. The central idea of principal component analysis is to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set [26]. This is achieved by transforming to a new set of variables, the principal components, which are uncorrelated, and which are ordered so that the first few retain most of the variation present in all of the original variables [26]. Orthogonal varimax rotation is used to achieve a strong correlation between each of the original variables and only one of the new principal components (simple structure). After rotation, the new principal components remain relatively independent and this rotation has no effect on the variation in the data that is explained by the new components.

For each principal component, the analysis generates an eigenvalue, which is the sum of squared correlations between the original variables and the principal component. The eigenvalue indicates the variance attributable to a principal component. Standardized variables have a variance equal to 1. An eigenvalue greater than 1 indicates that the corresponding principal component accounts for more total variance than the original standardized variables. Only principal components with an eigenvalue greater than 1 were selected in this analysis.

The inclusion of variables in a cluster depends on their factor loadings, which can be interpreted as the correlation coefficient between the original variable and the newly formed (principal) components. We used a factor loading of 0.40 as a marginal value, i.e. only factor loadings >0.40 are considered in the identification of a clustering pattern. This marginal value is arbitrarily chosen in such a manner that there will be little overlap between any of the newly formed components, thereby leading to an understandable interpretation of the results.

Since D-dimer, CRP and TG levels were not normally distributed they were natural log-transformed. Principal component analysis was performed first on hemostatic factors (procoagulant, anticoagulant and fibrinolytic factors), and second with the addition of CRP and lipid levels. All analyses were repeated excluding controls with malignancy or self -reported chronic diseases. Statistical analyses were performed with SPSS for Windows, release 23.0 (SPSS Inc, Chicago, IL).

RESULTS

Table 1 shows the characteristics of the study population. In the group of 2874 control subjects, the median age was 49.7 years (interquartile range 39.2-58.1 years], and 1353 (47.1%) were men. Levels of procoagulant, anticoagulant and fibrinolytic hemostatic factors, lipids and CRP are described in Table 1.

Table 1. Characteristics of the study population

Characteristics	Total study population	n = 2874
Demographic factors		
Male, n (%)	1353	(47.1)
Age (years)	49.7	(39.2-58.1)
Procoagulant factors		
Fibrinogen (g/L)	3.3	(0.7)
Factor II (IU/dL)	110.7	(15.3)
Factor V (U/mL)	0.93	(0.17)
Factor VII (IU/dL)	111.3	(24.7)
Factor VIII (IU/dL)	112.2	(38.2)
VWF (IU/dL)	111.4	(46.0)
Factor IX (IU/dL)	104.6	(18.4)
Factor X (IU/dL)	117.1	(18.6)
Factor XI (IU/dL)	100.2	(19.1)
Anticoagulant factors		
Antithrombin (IU/dL)	105.3	(10.7)
Protein C (IU/dL)	117.7	(21.1)
Protein S (IU/dL)	102.5	(19.7)
TFPI (U/dL)	174.5	(46.0)
Fibrinolytic factor		
D-dimer (ng/mL) †	236.6	(167.6-357.0)
Lipid profile		
Triglycerides (mmol/L) †	1.32	(1.00-1.87)
TC (mmol/L)	5.62	(1.11)
LDL-C (mmol/L)	3.56	(0.95)
HDL-C (mmol/L)	1.35	(0.39)
Apo A1 (g/L)	1.43	(0.29)
Apo B (g/L)	0.99	(0.26)
Inflammatory marker		
CRP (mg/L) †	1.43	(0.68-3.13)

Continuous variables are shown as mean (± standard deviation), or median (25th percentile - 75th percentile) when non-normally distributed (as marked with '†'). Categorical variables are shown as number (%).

Data were missing for some participants in some subgroups.

apo A1, apolipoprotein A1; apo B, apolipoprotein B; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TFPI, tissue factor pathway inhibitor; VWF, von Willebrand factor.

As depicted in Table 2, there were 3 clusters among the hemostatic factors with an eigenvalue greater than 1, which together accounted for more than 50% of the total variance. We identified a VKDF cluster (component 1 in Table 2), composed of all VKDFs (FII, FVII, FIX, FX, protein C, and protein S), that also included FXI and antithrombin. Another cluster (component 2 in Table 2) included FVIII, VWF,

Table 2. Principal component analysis with hemostatic factors in 2874 population controls. Factor loadings between the original variables and the newly formed components are shown

	Components		
Original variable	**1**	**2**	**3**
Procoagulant factors			
Fibrinogen	0.371	**0.556***	0.154
Factor II	**0.741***	-0.011	0.051
Factor V	0.147	0.175	**0.641***
Factor VII	**0.657***	0.125	0.164
Factor VIII	0.064	**0.818***	0.117
VWF	-0.003	**0.833***	0.096
Factor IX	**0.690***	0.298	-0.012
Factor X	**0.818***	0.021	-0.036
Factor XI	**0.517***	0.085	0.227
Anticoagulant factors			
Antithrombin	**0.415***	-0.301	**0.455***
Protein C	**0.748***	-0.042	0.226
Protein S	**0.475***	0.134	**0.456***
TFPI activity	-0.003	0.055	**0.785***
Fibrinolytic factor			
D-dimer†	0.034	**0.611***	-0.031
Variance			
% total variance	29.56	14.92	8.65
% cumulative variance	29.56	44.48	53.13

Data were missing for some participants in some subgroups.

The principal components were ranked according to their eigenvalues from 1 to 3.

TFPI, tissue factor pathway inhibitor; VWF, von Willebrand factor.

*Factor Loading > 0.40.

†Log-transformed variable.

fibrinogen and D-dimer, and a third one (component 3 in Table 2) was comprised of TFPI, FV, protein S, and antithrombin. Exclusion of control subjects with malignancy or self-reported chronic diseases yielded virtually the same clustering pattern, with the exception of antithrombin that was no longer included in the VKDF cluster (Table S1).

The inclusion of lipid fractions and CRP in the analysis led to three additional clusters with an eigenvalue greater than 1, now resulting in the formation of 6 principal components, which together accounted for almost 70% of the total variance. As shown in Table 3 and Fig. 1, there were two extra lipid clusters: one composed of TC, LDL-C, and apo B that also included TFPI (component 2 in Table 3), and another comprising HDL-C, apo A1 and TG that did not include any hemostatic factor (component 3 in Table 3). The VKDF cluster (component 1 in Table 3) now includes

Table 3. Principal component analysis with hemostatic factors, lipids and C-reactive protein in 2874 population controls. Factor loadings between the original variables and the newly formed components are shown

	Components					
Original variable	**1**	**2**	**3**	**4**	**5**	**6**
Procoagulant factors						
Fibrinogen	0.259	0.039	-0.083	**0.725***	0.159	0.268
Factor II	**0.675***	0.139	0.047	0.121	-0.080	0.131
Factor V	0.113	0.130	0.016	0.118	0.128	**0.625***
Factor VII	**0.656***	0.192	0.102	0.056	0.145	0.093
Factor VIII	0.087	0.003	0.044	0.171	**0.907***	0.098
VWF	0.017	0.061	-0.007	0.190	**0.909***	0.047
Factor IX	**0.649***	0.059	-0.110	**0.418***	0.096	0.031
Factor X	**0.794***	0.083	0.035	0.131	-0.032	0.007
Factor XI	**0.468***	-0.030	0.116	0.108	0.031	**0.409***
Anticoagulant factors						
Antithrombin	**0.452***	-0.057	-0.032	-0.375	-0.073	**0.542***
Protein C	**0.746***	0.212	0.103	-0.084	0.052	0.183
Protein S	**0.416***	0.204	-0.161	0.120	0.073	**0.451***
TFPI	-0.067	**0.403***	-0.066	0.075	0.002	**0.612***
Fibrinolytic factor						
D-dimer†	-0.087	0.021	0.093	**0.649***	0.218	0.091
Lipid profile						
Triglycerides†	**0.547***	0.365	**-0.460***	0.003	0.107	-0.155
TC	0.273	**0.911***	0.204	-0.005	0.048	0.136
LDL-C	0.133	**0.928***	-0.012	0.019	0.012	0.203
HDL-C	-0.003	-0.026	**0.970***	-0.046	0.019	0.007
Apo A1	0.209	0.046	**0.913***	-0.004	0.045	-0.101
Apo B	0.252	**0.893***	-0.197	0.075	0.026	0.116
Inflammatory marker						
CRP†	0.336	0.019	-0.097	**0.794***	0.036	-0.098
Variance						
% total variance	26.62	11.45	10.66	9.07	6.12	5.14
% cumulative variance	26.62	38.07	48.73	57.80	63.92	69.06

Data were missing for some participants in some subgroups.

The principal components were ranked according to their eigenvalues from 1 to 6.

apo A1, apolipoprotein A1; apo B, apolipoprotein B; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TFPI, tissue factor pathway inhibitor; VWF, von Willebrand factor.

*Factor Loading > 0.40.

†Log-transformed variables.

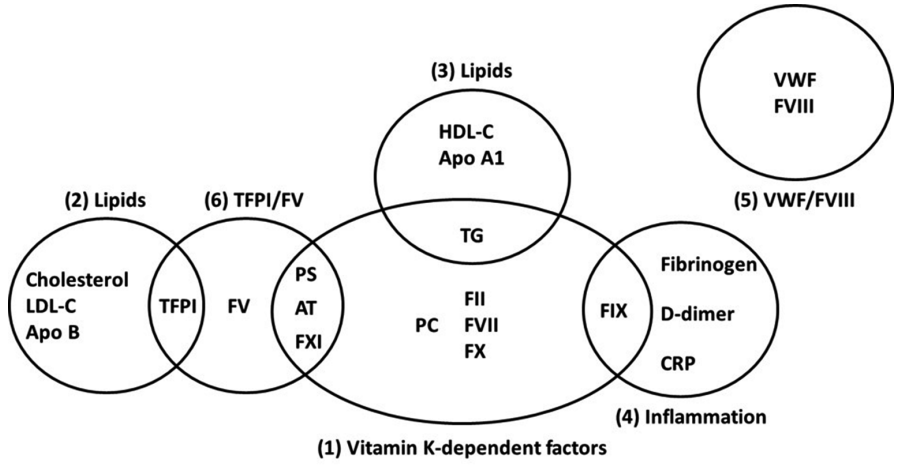

Figure 1. Factor loading pattern of hemostatic factors, lipids and C-reactive protein in 2874 population controls from the the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study. The principal components are represented by ellipses, and were ranked according to their eigenvalues from 1 to 6 (numbers between parentheses). AT, antithrombin; apo A1, apolipoprotein A1; apo B, apolipoprotein B; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PC, protein C; PS, protein S; TFPI, tissue factor pathway inhibitor; TG, triglycerides; VWF, von Willebrand factor.

TG. FVIII and WVF formed a new separate cluster (component 5 in Table 3), whereas CRP levels clustered with fibrinogen, D-dimer, and factor IX levels (component 4 in Table 3). The cluster composed of TFPI, FV, protein S, and antithrombin (component 6 in Table 3) remained practically unchanged, with the addition of FXI only. The exclusion of individuals with malignancy or self-reported chronic diseases resulted in minor changes in the clustering pattern, in which the 6 principal components also accounted for almost 70% of the total variance (Tables S2).

DISCUSSION

In this study, we investigated the interrelation between levels of hemostatic factors, lipids and CRP in 2874 population controls. We included several procoagulant and anticoagulant hemostatic factors and lipids, which enabled us to perform a comprehensive analysis on their clustering pattern. Among our main findings is that VKDFs, including procoagulant (FII, FVII, FIX, and FX) and anticoagulant (protein C and protein S) factors, clustered together. Upon addition of lipids to the analysis, all VKDFs consistently clustered with TG levels. Furthermore, we found that CRP levels clustered together with fibrinogen, D-dimer, and FIX levels. The clustering patterns

observed in this study suggest that there might be common mechanisms regulating hemostatic factor and lipid levels, and the inflammatory response. Whether and to what extent the interrelation between levels of hemostatic factors, lipids and CRP contributes to the risk of venous thrombosis and arterial CVD remains unsettled. This is a relevant issue from both a mechanistic and a clinical viewpoint, as therapeutic strategies targeting possible common regulatory mechanisms of hemostatic factors and cardiometabolic risk factors, like statins [19,27], have the potential to decrease the risk of both venous thrombosis and arterial CVD. Indeed, experimental data have demonstrated that statins may have antithrombotic effects that are unrelated to their lipid-lowering activity [27].

Since the liver is the main site of production of coagulation factors [28], it is biologically plausible that the interrelation between hemostatic factors could be explained, at least in part, by common mechanisms regulating their biosynthesis. For instance, hepatocyte nuclear factor 4 (HNF-4) is a transcription factor highly expressed in the liver, that has been shown to regulate the expression of several genes, such as those encoding FII, FVII, FIX, FX, and FXI, and anticoagulant factors protein S and antithrombin [29-35]. Therefore, one may consider that common determinants in the regulation of the transcription of hemostatic factors, such as HNF-4, could contribute to the clustering of VKDF, FXI and antithrombin levels, as depicted in Fig. 1. The clustering of VKDFs could be further explained by a fundamental post-translational step during the biosynthesis of these factors. This step is a vitamin K-dependent enzymatic reaction mediated by γ-glutamyl carboxylase, which leads to the conversion of glutamic acid to γ- carboxyglutamic acid [36]. The modification of glutamic acid residues enables these factors to bind to phospholipid membranes at physiological calcium concentration [37], which is key for their activity [38]. Although previous studies have also shown that VKDFs clustered together, including the Leiden Thrombophilia Study (LETS) [2], not all factors were measured in these studies [1], or showed a consistent clustering pattern between all procoagulant and anticoagulant VKDFs [1-3], as observed in the present analysis.

Here we found that TG, but not the other lipids studied, clustered with VKDFs. This result is in line with a previous finding from Sakkinen *et al*. [1], in which TG levels clustered together with FVII, FIX and FX in the Cardiovascular Health Study that included 322 elderly participants. Vanschoonbeek *et al*. [4] also described clustering between VKDF (FII, FVII, and FX) and TG levels in a study comprising 57 overweight men and 42 overweight individuals with type 2 diabetes. Interestingly, results from an animal model study also pointed to a relationship between VKDF and TG levels. In this study, which involved a murine model of type III hyperlipidemia, mice kept on fish oil diet (n-3 polyunsaturated fatty acids [n-3 PUFAs]) for 21 days had a reduction in plasma triglyceride levels, thrombin generation, and activity of VKDFs, but not in

VKDF mRNA levels, which remained unchanged [39]. Analysis of mouse livers showed that n-3 PUFA was associated with upregulation of genes related to lipid degradation, and downregulation of genes related to lipid synthesis and of γ-glutamyl carboxylase. The latter gene encodes the enzyme responsible for the γ-carboxylation of VKDFs, which, as previously pointed out, is a fundamental post-translational step for the activity of these factors in blood coagulation [38]. On the basis of our and other results [1,4], and the murine model study [39], one may speculate that common mechanisms lying outside the genes coding for VKDFs and TG could explain, at least in part, the clustering between VKDFs and TG.

In the present study, TC, LDL-C and apo B levels clustered together, whereas HDL-C levels formed a separate cluster with apo A1 and TG levels. These clustering patterns seem logical, as apo B is the protein component of the very low-density/low-density lipoprotein spectrum, and apo A1 is the major protein component of HDL-C [40]. Of note, total TFPI activity levels clustered with TC, LDL-C, and apo B, which is consistent with the fact that the majority of the TFPI in plasma is bound to apo B-containing lipoproteins [41]. Importantly, TFPI and FV composed a cluster that also included total protein S and antithrombin. This clustering pattern is in line with previous findings showing a close relationship between TFPI, FV, and protein S levels [42-44]. In the study by Duckers *et al*. [42], TFPI levels were markedly reduced in individuals with severe FV deficiency as compared with healthy control subjects. In the same study, FV and TFPI levels were also found to be strongly correlated in both healthy and FV-deficient individuals [42]. Thereafter, Dahm *et al*. [43] showed by means of linear regression that protein S and FV levels were the strongest determinants of TFPI levels in healthy individuals from the LETS. In experimental studies, TFPI has been suggested to bind to FV in plasma [42], and protein S has been shown to be a cofactor of TFPI that facilitates optimal FXa-inhibition and efficient down-regulation of thrombin generation in plasma [44]. Finally, we have no explanation for the clustering of antithrombin with the aforementioned factors other than that they are inhibitors of the coagulation system (with the exception of FV).

FVIII, VWF, fibrinogen and D-dimer levels are known to increase during inflammatory disorders [45-48], and pro-inflammatory cytokines can up-regulate the expression of fibrinogen [46] and FVIII [45]. Therefore, our result on the clustering of these factors is biologically reasonable, as inflammation could be a common mechanism affecting their levels. However, upon addition of CRP to the analysis, FVIII and VWF levels formed a separate cluster, whereas CRP levels clustered together with fibrinogen, D-dimer, and FIX levels. In the study by Sakkinen *et al*. [1], CRP levels also clustered with fibrinogen, D-dimer, and factor IX levels, like we found, but in addition with FVIII and plasmin-α_2-antiplasmin levels. However, in their study, VWF was not assessed. In line with our results, Vossen *et al*. [3] also demonstrated that FVIII and

VWF composed a separate cluster. FVIII and VWF plasma levels are strongly related, as VWF regulates FVIII levels by acting as a carrier protein [49], and this could explain the formation of a separate cluster when an extra variable (i.e. CRP) was added to the analysis.

The strengths of this study include that to our knowledge this is the largest study that has been performed on this issue so far, in which levels of several hemostatic factors and lipids were measured. Moreover, the detailed knowledge of self-reported disease status enable us to repeat all analyses in a healthy population. The main limitation is related to our study design, i.e. we could investigate the interrelation between hemostatic factor, lipid and CRP levels but not the mechanisms, related to either genetic or environmental factors, underlying their interrelation.

In conclusion, in this comprehensive study, we confirmed and extended clustering patterns of previous reports between levels of hemostatic factors, lipids and CRP.

REFERENCES

1. Sakkinen PA, Wahl P, Cushman M, Lewis MR, Tracy RP. Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome. Am J Epidemiol 2000; 152: 897-907.
2. Van Hylckama Vlieg A, Callas PW, Cushman M, Bertina RM, Rosendaal FR. Inter-relation of coagulation factors and d-dimer levels in healthy individuals. J Thromb Haemost 2003; 1: 516-22.
3. Vossen CY, Callas PW, Hasstedt SJ, Long GL, Rosendaal FR, Bovill EG. A genetic basis for the interrelation of coagulation factors. J Thromb Haemost 2007; 5: 1930-5.
4. Vanschoonbeek K, Feijge MA, Saris WH, de Maat MP, Heemskerk JW. Plasma triacylglycerol and coagulation factor concentrations predict the anticoagulant effect of dietary fish oil in overweight subjects. J Nutr 2007; 137: 7-13.
5. Doggen CJ, Berckmans RJ, Sturk A, Manger Cats V, Rosendaal FR. C-reactive protein, cardiovascular risk factors and the association with myocardial infarction in men. J Intern Med 2000; 248: 406-14.
6. Sørensen HT, Horvath-Puho E, Pedersen L, Baron JA, Prandoni P. Venous thromboembolism and subsequent hospitalisation due to acute arterial cardiovascular events: a 20-year cohort study. Lancet 2007; 370: 1773-9.
7. Becattini C, Vedovati MC, Ageno W, Dentali F, Agnelli G. Incidence of arterial cardiovascular events after venous thromboembolism: a systematic review and a meta-analysis. J Thromb Haemost 2010; 8: 891-7.
8. Roach RE, Lijfering WM, Flinterman LE, Rosendaal FR, Cannegieter SC. Increased risk of CVD after VT is determined by common etiologic factors. Blood 2013; 121: 4948-54.
9. Lind C, Flinterman LE, Enga KF, Severinsen MT, Kristensen SR, Braekkan SK, Mathiesen EB, Njølstad I, Cannegieter SC, Overvad K, Hansen JB. Impact of incident venous thromboembolism on risk of arterial thrombotic diseases. Circulation 2014; 129: 855-63.
10. Ljungqvist M, Holmström M, Kieler H, Odeberg J, Lärfars G. Cardiovascular disease and mortality after a first episode of venous thromboembolism in young and middle-aged women. Thromb Res 2016; 138: 80-5.
11. Lijfering WM, Flinterman LE, Vandenbroucke JP, Rosendaal FR, Cannegieter SC.Relationship between venous and arterial thrombosis: a review of the literature from a causal perspective. Semin Thromb Hemost 2011; 37: 885-96.
12. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. JAMA 2009; 302: 1993-2000.
13. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997; 336: 973-9.
14. Haslam DW, James WP. Obesity. Lancet 2005; 366: 1197-1209.
15. Ageno W, Becattini C, Brighton T, Selby R, Kamphuisen PW. Cardiovascular risk factors and venous thromboembolism: a meta-analysis. Circulation 2008; 117: 93-102.

16. Folsom AR, Lutsey PL, Astor BC, Cushman M. C-reactive protein and venous thromboembolism. A prospective investigation in the ARIC cohort. Thromb Haemost 2009; 102: 615-9.

17. Horvei LD, Grimnes G, Hindberg K, Mathiesen EB, Njølstad I, Wilsgaard T, Brox J, Braekkan SK, Hansen JB. C-reactive protein, obesity, and the risk of arterial and venous thrombosis. J Thromb Haemost 2016; 14: 1561-71.

18. Braekkan SK, Siegerink B, Lijfering WM, Hansen JB, Cannegieter SC, Rosendaal FR. Role of obesity in the etiology of deep vein thrombosis and pulmonary embolism: current epidemiological insights. Semin Thromb Hemost 2013; 39: 533-40.

19. Lijfering WM, Biedermann JS, Kruip MJ, Leebeek FW, Rosendaal FR, Cannegieter SC. Can we prevent venous thrombosis with statins: an epidemiologic review into mechanism and clinical utility. Expert Rev Hematol 2016; 9: 1023-30.

20. van Stralen KJ, Rosendaal FR, Doggen CJ. Minor injuries as a risk factor for venous thrombosis. Arch Intern Med 2008; 168: 21-26.

21. Meltzer ME, Lisman T, de Groot PG, Meijers JC, le Cessie S, Doggen CJ, Rosendaal FR. Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. Blood 2010; 116: 113-121.

22. Timp JF, Lijfering WM, Flinterman LE, van Hylckama Vlieg A, le Cessie S, Rosendaal FR, Cannegieter SC. Predictive value of factor VIII levels for recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2015; 13: 1823-32.

23. Rezende SM, Lijfering WM, Rosendaal FR, Cannegieter SC. Hematologic variables and venous thrombosis: red cell distribution width and blood monocyte count are associated with an increased risk. Haematologica 2014 ; 99: 194-200.

24. Morelli VM, Lijfering WM, Rosendaal FR, Cannegieter SC. Lipid levels and risk of recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2017; 15: 695-701.

25. Clauss A. Rapid physiological coagulation method in determination of fibrinogen [in German]. Acta Haematol 1957; 17: 237-246.

26. Jolliffe IT. Principal Component Analysis. New York: Springer; 2002.

27. Violi F, Calvieri C, Ferro D, Pignatelli P. Statins as antithrombotic drugs. Circulation 2013; 127: 251-7.

28. Brummel-Ziedins K, Orfeo T, Jenny NS, et al. Blood coagulation and fibrinolysis. In: Greer JP, Foerster J, Rodgers GM, Paraskevas F, Glader B, Arber DA, Means RT, eds. Wintrobe's Clinical Hematology. Philadelphia: Lippincott Williams & Wilkins; 2009: 528-619.

29. Ceelie H, Spaargaren-Van Riel CC, de Jong M, Bertina RM, Vos HL. Functional characterization of transcription factor binding sites for HNF1-alpha,HNF3-beta (FOXA2),HNF4-alpha, Sp1 and Sp3 in the human prothrombin gene enhancer. J Thromb Haemost 2003; 1: 1688– 98.

30. Erdmann D, Heim J. Orphan nuclear receptor HNF-4 binds to the human coagulation factor VII promoter. J Biol Chem 1995; 270: 22988–96.

31. Reijnen MJ, Peerlinck K, Maasdam D, Bertina RM, Reitsma PH. Hemophilia B Leyden: substitution of thymine for guanine at position-21 results in a disruption of a hepatocyte nuclear factor 4 binding site in the factor IX promoter. Blood 1993; 82: 151–8.

32. Hung HL, High KA. Liver-enriched transcription factor HNF-4 and ubiquitous factor NF-Y are critical for expression of blood coagulation factor X. J Biol Chem 1996; 271: 2323-31.

33. Tarumi T, Kravtsov DV, Zhao M, Williams SM, Gailani D. Cloning and characterization of the human factor XI gene promoter: Transcription factor HNF-4alpha is required for hepatocyte specific expression of factor XI. J Biol Chem 2002; 277: 18510–6.

34. Hall AJ, Peake IR, Winship PR. Regulation of the human protein S gene promoter by liver enriched transcription factors. Br J Haematol 2006; 135: 538–46.

35. Fernandez-Rachubinski FA, Weiner JH, Blajchman MA. Regions flanking exon 1 regulate constitutive expression of the human antithrombin gene. J Biol Chem 1996; 271: 29502-12.

36. Furie B, Bouchard BA, Furie BC. Vitamin K-dependent biosynthesis of gamma carboxyglutamic acid. Blood 1999; 93: 1798-808.

37. Nelsestuen GL, Broderius M, Zytkovicz TH, Howard JB. On the role of gamma carboxyglutamic acid in calcium and phospholipid binding. Biochem Biophys Res Commun 1975; 65: 233-40.

38. Oldenburg J, Watzka M, Rost S, Muller CR. VKORC1: molecular target of coumarins. J Thromb Haemost 2007; 5: 1-6.

39. Vanschoonbeek K, Wouters K, van der Meijden PE, van Gorp PJ, Feijge MA, Herfs M, Schurgers LJ, Hofker MH, de Maat MP, Heemskerk JW. Anticoagulant effect of dietary fish oil in hyperlipidemia: a study of hepatic gene expression in APOE2 knock-in mice. Arterioscler Thromb Vasc Biol 2008; 28: 2023-29.

40. Marcovina S, Packard CJ. Measurement and meaning of apolipoprotein AI and apolipoprotein B plasma levels. J Intern Med 2006; 259: 437-446.

41. Crawley JT, Lane DA. The haemostatic role of tissue factor pathway inhibitor. Arterioscler Thromb Vasc Biol 2008; 28: 233-242.

42. Duckers C, Simioni P, Spiezia L, Radu C, Gavasso S, Rosing J, Castoldi E. Low plasma levels of tissue factor pathway inhibitor in patients with congenital factor V deficiency. Blood 2008; 112: 3615-23.

43. Dahm AEA, Bezemer ID, Sandset PM, Rosendaal FR. Interaction between tissue factor pathway inhibitor and factor V levels on the risk of venous thrombosis. J Thromb Haemost 2010; 8: 1130-2.

44. Hackeng TM, Maurissen LFA, Castoldi E, Rosing J. Regulation of TFPI function by protein S. J Thromb Haemost 2009; 7: 165-8.

45. Kerr R, Stirling D, Ludlam CA. Interleukin 6 and haemostasis. Br J Haematol 2001; 115: 3-12.

46. Fish RJ, Neerman-Arbez M. Fibrinogen gene regulation. Thromb Haemost 2012; 108: 419-26.

47. Blann AD. Plasma von Willebrand factor, thrombosis, and the endothelium: the first 30 years. Thromb Haemost 2006; 95: 49-55.

48. Haapaniemi E, Tatlisumak T. Is D-dimer helpful in evaluating stroke patients? A systematic review. Acta Neurol Scand 2009; 119: 141-50.

49. Sadler JE. Biochemistry and genetics of von Willebrand factor. Annu Rev Biochem 1998; 67: 395-424.

SUPPLEMENTAL MATERIAL

Supplementary Table 1. Principal component analysis with hemostatic factors in 2304 population controls without malignancy or self-reported chronic diseases. Factor loadings between the original variables and the newly formed components are shown

	Components		
Original variable	**1**	**2**	**3**
Procoagulant factors			
Fibrinogen	0.384	**0.542***	0.132
Factor II	**0.797***	0.008	0.077
Factor V	0.130	0.184	**0.642***
Factor VII	**0.641***	0.085	0.189
Factor VIII	0.033	**0.833***	0.152
VWF	-0.004	**0.845***	0.135
Factor IX	**0.696***	0.273	-0.021
Factor X	**0.825***	0.025	-0.054
Factor XI	**0.515***	0.072	0.209
Anticoagulant factors			
Antithrombin	0.375	-0.326	**0.469***
Protein C	**0.734***	-0.041	0.240
Protein S	**0.442***	0.091	**0.482***
TFPI activity	-0.004	0.062	**0.768***
Fibrinolytic factor			
D-dimer†	0.063	**0.596***	-0.070
Variance			
% total variance	29.23	15.19	9.02
% cumulative variance	29.23	44.42	53.44

Data were missing for some participants in some subgroups.

The principal components were ranked according to their eigenvalues from 1 to 3.

TFPI, tissue factor pathway inhibitor; VWF, von Willebrand factor.

*Factor Loading > 0.40.

†Log-transformed variable.

Supplementary Table 2. Principal component analysis with hemostatic factors, lipids and C-reactive protein in 2304 population controls without malignancy or self-reported chronic diseases. Factor loadings between the original variables and the newly formed components are shown

	Components					
Original variable	**1**	**2**	**3**	**4**	**5**	**6**
Procoagulant factors						
Fibrinogen	0.249	0.062	-0.081	**0.724***	0.159	0.257
Factor II	**0.734***	0.132	0.040	0.145	-0.057	0.166
Factor V	0.079	0.165	0.028	0.108	0.154	**0.626***
Factor VII	**0.630***	0.253	0.150	0.020	0.129	0.090
Factor VIII	0.064	0.003	0.044	0.169	**0.921***	0.097
VWF	0.024	0.062	-0.009	0.192	**0.919***	0.052
Factor IX	**0.636***	0.091	-0.093	**0.429***	0.071	0.026
Factor X	**0.802***	0.085	0.018	0.148	-0.024	-0.013
Factor XI	**0.455***	-0.030	0.107	0.125	0.004	**0.425***
Anticoagulant factors						
Antithrombin	**0.441***	-0.070	-0.060	**-0.404***	-0.076	**0.536***
Protein C	**0.731***	0.227	0.106	-0.082	0.060	0.181
Protein S	0.366	0.234	-0.177	0.112	0.036	**0.467***
TFPI	-0.087	**0.422***	-0.078	0.072	0.014	**0.588***
Fibrinolytic factor						
D-dimer†	-0.087	0.008	0.125	**0.626***	0.214	0.096
Lipid profile						
Triglycerides†	**0.538***	**0.405***	**-0.449***	0.005	0.071	-0.153
TC	0.283	**0.907***	0.192	0.005	0.037	0.148
LDL-C	0.150	**0.919***	-0.032	0.021	0.013	0.217
HDL-C	-0.002	-0.048	**0.969***	-0.029	0.021	0.002
Apo A1	0.212	0.034	**0.912***	0.015	0.034	-0.106
Apo B	0.262	**0.886***	-0.196	0.074	0.032	0.129
Inflammatory marker						
CRP†	0.345	0.014	-0.097	**0.791***	0.042	-0.095
Variance						
% total variance	27.03	11.60	10.59	9.01	6.16	5.09
% cumulative variance	27.03	38.63	49.22	58.23	64.39	69.48

Data were missing for some participants in some subgroups.

The principal components were ranked according to their eigenvalues from 1 to 6.

apo A1, apolipoprotein A1; apo B, apolipoprotein B; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TFPI, tissue factor pathway inhibitor; VWF, von Willebrand factor.

*Factor Loading > 0.40.

†Log-transformed variables.

Chapter 6

Association between hepatic triglyceride content and coagulation factor levels: The Netherlands Epidemiology of Obesity Study

V.M. Morelli, R. de Mutsert, A. de Roos, H.J. Lamb, A. van Hylckama Vlieg, M.H.A. Bos, F. R. Rosendaal, W.M. Lijfering, S.C. Cannegieter

To be submitted

ABSTRACT

Background

Hepatic triglyceride content (HTGC) has been associated with levels of coagulation factors, which is relevant to improve understanding of the pathogenesis of venous thrombosis (VT). However, it is unclear whether HTGC contributes to coagulation factor levels beyond total body fat (TBF) and visceral adipose tissue (VAT).

Objective

To investigate the association between HTGC and levels of fibrinogen, and factors (F) VIII, IX and XI while taking into account TBF and VAT.

Methods

This is a cross-sectional analysis in a subset of participants of the NEO study (n=6671) who underwent magnetic resonance (MR) imaging and MR spectroscopy to assess VAT and HTGC (n=2580). We excluded participants without complete imaging and coagulation factor assessment, with liver disease, a history of VT, or on anticoagulation.

Results

1946 participants were included (53% women; median age 56 years). Coagulation factor levels increased dose-dependently across HTGC quartiles in linear regression models adjusted for age, sex, ethnicity, education, alcohol intake, physical activity, smoking, estrogen, and menopause. Mean differences between the fourth and first (reference) quartiles were 14.4 mg/dL (95% CI: 1.8, 26.9) for fibrinogen, 6.6 IU/dL (95% CI: 0.4, 12.8) for FVIII, 26.1 IU/dL (95% CI: 22.4, 29.8) for factor IX, and 8.4 IU/dL (95% CI 4.4, 12.5) for FXI. With further adjustment for TBF and VAT, the dose-response association of HTGC with factor IX levels persisted, whereas associations with the other factors disappeared.

Conclusions

HTGC was associated with levels of various coagulation factors, of which FIX remained associated with HTGC after adjustment for TBF and VAT. HTGC has the potential to contribute to VT risk beyond total body and visceral fat through FIX levels.

INTRODUCTION

The association between obesity and venous thrombosis is well established in epidemiological studies [1]. However, the mechanism underlying this association is not fully understood, and probably reflects the coexistence of multiple pathophysiological pathways. In particular, excess fat accumulation in the liver, also referred to as nonalcoholic fatty liver disease (NAFLD), is strongly associated with obesity and insulin resistance [2], and could be one of the mechanisms by which obesity increases the risk of venous thrombosis. NAFLD is a term used to describe a broad range of related disorders, initiating from simple steatosis (accumulation of triglycerides as lipid droplets in the cytoplasm of hepatocytes), which may progress to nonalcoholic steatohepatitis (NASH) (steatosis associated with inflammation, hepatocyte injury, and/or fibrosis), cirrhosis (replacement of hepatocytes by scar tissue), and liver cancer (hepatocellular carcinoma) [2]. NAFLD is a common disease, occurring in approximately 20 to 30% of adults in the general population in Western countries [3]. Its prevalence increases to 70 to 90% among those who are obese or have type 2 diabetes. The role of NAFLD in the risk of venous thrombosis is as yet largely unknown. In one previous study composed of 138 patients with unprovoked venous thrombosis and 276 controls, the prevalence of NAFLD was almost 3-fold higher in cases (81%) than in controls (30%) [4].

In the past two decades, several small studies have investigated the association between NAFLD and hemostatic factors, mainly in relation to plasminogen activator inhibitor-1 [5-19], a key inhibitor of the fibrinolytic system. However, results of these studies have often been inconsistent, probably due to limited statistical power of the individual studies and differences in study design, clinical characteristics of the study population, methods used to define NAFLD (liver biopsy vs. radiological imaging), or adjustments for potential confounding factors. It is of interest that in one of these studies (n=98) [18], liver fat content was positively correlated with levels of coagulation factors that are associated with an increased risk of venous thrombosis, i.e., factors VIII, IX and XI [20], independent of age, sex and body mass index (BMI). However, the amount of abdominal visceral adipose tissue (VAT) was not taken into account in the relationship between liver fat content and the aforementioned coagulation factors. VAT might be an important confounding factor, as it is strongly related to liver fat [21,22], while it has also been shown to be related to levels of some coagulation factors [6,23]. Moreover, since VAT is highly associated with total body fat (TBF) [24,25], adjustment for TBF should be performed when studying specific effects of VAT [26]. Hence, whether liver fat content is associated with levels of coagulation factors beyond total body and visceral fat is as yet unclear. Since obesity and fatty liver are potentially modifiable through lifestyle intervention [3], clarification of this question is a relevant clinical issue worth pursuing.

Localized hydrogen 1 (^{1}H) magnetic resonance spectroscopy (MRS) is a sensitive, quantitative, and noninvasive method for determining liver fat content, measured as hepatic triglyceride content (HTGC) [27]. The aim of our study was to investigate the association between HTGC, assessed by ^{1}H MRS, and levels of fibrinogen, and factors VIII, IX, and XI. We further hypothesized that this association could be explained, at least in part, by common causes (confounding variables), of both HTGC and coagulation factor levels, such as demographic and lifestyle factors [3,28-32], and total body and visceral fat [6,21-23]. As sex differences are observed in body fat distribution [33], liver fat content [22,34,35], and risk of venous thrombosis [36], we additionally performed subgroup analyses stratified by sex. For these purposes, we performed a cross-sectional analysis in the Netherlands Epidemiology of Obesity (NEO) study.

METHODS

Study design and study population

The NEO study is a population-based cohort study designed to investigate pathways that lead to obesity-related diseases. The NEO study includes 6671 participants, with an oversampling of individuals with overweight or obesity. Details on the design and study population were described elsewhere [37]. Briefly, between September 2008 and September 2012, men and women aged 45-65 years with a self-reported BMI of 27 kg/m^2 or higher living in the greater area of Leiden (in the West of The Netherlands) were eligible to participate in the NEO study. In addition, all inhabitants aged 45-65 years from one municipality (Leiderdorp) were invited to participate, irrespective of their BMI, in order to obtain a reference distribution of BMI.

The present study is a cross-sectional analysis of the baseline data from the NEO study. At the time of inclusion, a screening form was completed by all participants asking about contraindications to magnetic resonance imaging (MRI) [37]. Among the eligible participants, 2580 were randomly selected to undergo ^{1}H MRS to assess HTGC, and MRI to assess abdominal subcutaneous and visceral fat [37,38]. Of these, 2075 participants had complete imaging and quantification of HTGC, and of abdominal subcutaneous and visceral fat depots.We subsequently excluded participants with missing data on levels of coagulation factors (n=27), with a known history of liver disease (n=25) or venous thrombosis (n=53), or who reported anticoagulant treatment at the time of blood sampling (n=24), thus leaving 1946 participants for the current analysis. The NEO study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC), and all participants gave written informed consent.

Data collection and blood sampling

Participants were invited to visit the NEO study center after an overnight fast for baseline measurements, including blood sampling and anthropometry [37]. Prior to the baseline visit, participants completed questionnaires at home on demographic, lifestyle and clinical data [37]. In this study, we grouped demographic and lifestyle factors as follows: ethnicity into white and other, the level of education into high and other (according to the Dutch education system, participants with higher secondary education, higher vocational education, university, and PhD were categorized as highly educated), tobacco smoking into current and other (never and former smoker), estrogen use into current and other (never and former user), and menopause status into postmenopausal and premenopausal. Physical activity was expressed in metabolic equivalents of task (MET)-hours per week, and alcohol consumption was expressed as a continuous (g/day) or a categorical variable (<10g/day, 10-20g/day, 20-40g/day, and ≥40g/day).

Measurements of body fat and magnetic resonance studies

Body weight was measured and TBF (%) was estimated by the Tanita bio impedance balance (TBF-310, Tanita International Division, UK) [37]. BMI was calculated by dividing the weight in kilograms by the height in meters squared. Waist circumference (WC) was measured mid-way between the lower costal margin and the iliac crest (cm).

MR imaging and spectroscopy were performed with a 1.5-T whole-body MR unit (Philips Medical Systems, Best, the Netherlands), details of which have been described elsewhere [26,37,38]. Briefly, abdominal VAT and subcutaneous adipose tissue (SAT) areas were quantified with a turbo spin-echo MRI protocol. At the level of the fifth lumbar vertebra, three transverse images each with a slice thickness of 10 mm were obtained during one breath-hold. Abdominal SAT and VAT were quantified by converting the number of pixels to square cm (MASS, Medis, Leiden, the Netherlands), and the average of the three slices was used for analyses.

HTGC was determined by ^{1}H MRS, as previously described in the NEO study [38]. In short, an 8-mL voxel was positioned in the right lobe of the liver. A point-resolved spectroscopy sequence was used to acquire spectroscopic data during continuous breathing with automated shimming. Spectra were obtained with and without water suppression. The resonances that were fitted and used for calculation of the triglycerides were methylene and methyl. The HTGC relative to water was calculated with the following formula: (signal amplitude of methylene + methyl) / (signal amplitude of water) x 100.

Laboratory measurements

Blood samples for coagulation factor measurements were drawn into tubes containing 0.106M trisodium citrate (Sarstedt, Nümbrecht, Germany). Plasma was obtained by centrifugation at 2500*g* for 10 min at room temperature and stored in aliquots at -80°C until testing. Fibrinogen activity was measured according to the method of Clauss [39]. Factor VIII activity, factor IX activity and factor XI activity were measured with a mechanical clot detection method on an ACL TOP 700 analyzer (Werfen, Barcelona, Spain). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by ultraviolet tests on a Cobas Integra 800 analyzer (Roche Diagnostics, Mannheim, Germany) [37]. All assays were performed by laboratory technicians who were unaware of the status of the samples.

Statistical analyses

In the NEO study, there is an oversampling of individuals with a BMI of 27 kg/m^2 or higher. As previously described [26], to correctly represent baseline associations in the general population, adjustments for the oversampling of individuals with a BMI ≥ 27 kg/m^2 were made. This was done by weighting individuals towards the BMI distribution of participants from the Leiderdorp municipality, whose BMI distribution was similar to the BMI distribution of the general Dutch population aged 45-65 years. All results are based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of individuals with a BMI ≥ 27 kg/m^2.

Baseline characteristics of the weighted study population are expressed as proportion instead of absolute numbers for categorical variables, and as mean (± standard deviation [SD]) or median (25th and 75th percentiles) for continuous variables.

Association between body fat measures and HTGC

We used linear regression to obtain insight into the degree to which the measures of body fat and body fat distribution (BMI, TBF, WC, VAT, abdominal SAT, and VAT/SAT ratio) were associated with HTGC. Because the distribution of HTGC and VAT/SAT ratio was skewed to the right, natural log-transformation was applied for both variables in all analyses (ln HTGC and ln VAT/SAT ratio). We calculated Z-scores of body fat measures to standardize the values of these measures to a mean of zero and a SD of one.

Weighted linear regression analyses were performed to assess the associations between each standardized measure of body fat (independent variable) and ln HTGC (dependent variable). The resulting regression coefficient (β) and its 95% confidence intervals (CIs) for a measure of body fat indicates the difference in ln HTGC when

that particular measure increases by one SD. We constructed scatter plots between Z-scores of body fat measures and ln HTGC, and observed that the assumption of linearity was met in all regression models. Crude associations (model 1) were adjusted for age and sex in model 2, and in model 3 for age, sex and for the other potential confounding factors, i.e., ethnicity (dichotomous value), education level (dichotomous value), alcohol intake (continuous value), physical activity (continuous value), tobacco smoking (dichotomous value), estrogen use (dichotomous value), and menopause status (dichotomous value).

Association between HTGC and coagulation factor levels

We calculated Z-scores of ln HTGC, and used weighted linear regression to examine the increase or decrease in levels of each coagulation factor (dependent variable) per one SD increase in ln HTGC (independent variable). Levels of coagulation factors were normally distributed and assumption of linearity was met in all regression models. Crude associations (model 1), were adjusted for age and sex (model 2), and further for the potential confounding factors described in model 3, adding VAT (continuous value) and TBF (continuous value) in a fourth model. We also investigated whether the association between HTGC and coagulation factor levels followed a dose-response relation. For this purpose, we *a-priori* categorized ln HTGC into quartiles, and used weighted linear regression to estimate mean differences and their 95% CIs in levels of coagulation factors for the second, third, and fourth quartile of ln HTGC compared with the first quartile (reference category). The regression coefficient for a ln HTGC quartile indicates the mean difference in levels of coagulation factors between that particular quartile and the reference category. We adjusted associations for the same aforementioned confounding factors described in models 2, 3 and 4. In subgroup analyses, we assessed the relationship between ln HTGC quartiles and levels of coagulation factors stratified by sex.

All analyses were repeated excluding participants with alcohol consumption ≥ 20g/day at the baseline study visit (sensitivity analyses). Statistical analyses were performed with STATA Statistical Software, version 12.0 (Statacorp, College Station, Texas, USA).

RESULTS

Baseline characteristics

Table 1 shows the baseline characteristics of the 1946 participants, of whom 53% were women. The median age was 56 years (interquartile range [IQR] 50, 61), 96% of participants were white, 47% were highly educated, and 14% were current smokers. Median alcohol consumption was 10.4 g/day (IQR 2.8, 21.4), and median physical

Table 1. Baseline characteristics of 1946 participants from the NEO study

Characteristics	
Demographic and lifestyle factors	
Sex (% women)	53
Age (years)	56 (50, 61)
Ethnicity (% whites)	96
Education level (% high)[a]	47
Alcohol consumption (g/day)	10.4 (2.8, 21.4)
Alcohol consumption (%)	
<10g/day	49
10-20g/day	21
20-40g/day	22
≥40g/day	8
Physical activity (MET-hours per week)	30.2 (15.8, 51.5)
Tobacco smoking (% current)	14
Estrogen (% current use, in women)	10
Menopause status (% postmenopausal, in women)	81
Measures of adiposity	
BMI (kg/m^2)	25.9 (3.9)
Total body fat (%)	30.7 (8.3)
Waist circumference (cm)	91.1 (12.6)
VAT (cm^2)	88.3 (54.5)
SAT (cm^2)	232.6 (96.5)
VAT/SAT	0.34 (0.22, 0.52)
Hepatic triglyceride content (%)	2.66 (1.34, 6.27)
Coagulation factors	
Fibrinogen (mg/dL)	289 (55)
Factor VIII (IU/dL)	122 (32)
Factor IX (IU/dL)	116 (20)
Factor XI (IU/dL)	116 (20)
Transaminases	
ALT (U/L)	25.1 (11.5)
AST (U/L)	24.7 (8.1)

Results were based on analyses weighted towards a normal body mass index distribution. Data were missing for some participants in some subgroups. Data are shown as mean (± standard deviation), median (25th percentile -75th percentile) or percentage. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; MET, metabolic equivalents of task; SAT, abdominal subcutaneous adipose tissue; VAT, visceral adipose tissue; VAT/SAT, ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue; WC, waist circumference. [a] High educational level (according to Dutch educational system): higher secondary education, higher vocational education, university, and PhD.

activity during leisure time was 30.2 MET-hours per week (IQR 15.8, 51.5). Among women, 81% referred a postmenopausal status and 10% were current users of estrogen. Participants had a mean BMI of 25.9 kg/m^2 ± 3.9, and a median HTGC of 2.66% (IQR 1.34, 6.27). Supplementary Table 1 describes the baseline characteristics

separately in men and women. Men had higher WC, VAT, HTGC and transaminases levels, and lower abdominal SAT and TBF than women. When participants with alcohol consumption ≥20g/day were excluded (n=620) (Table S2), the proportion of men, current smokers and highly educated participants, and the amount of VAT and HTGC were slightly lower as compared with subjects included in the main analysis, with no substantial differences in the other variables.

Association between body fat measures and HTGC

Table 2 shows that all measures of body fat and body fat distribution were associated with ln HTGC, and upon adjustment for potential demographic and lifestyle confounding factors (model 3), the strongest associations were observed for TBF, VAT and WC. When participants with alcohol consumption ≥20g/day were excluded, results were virtually the same (Table S3).

Association between HTGC and coagulation factor levels

Associations between ln HTGC and coagulation factor levels are described in Table 3. For reporting and interpretation, values of ln HTGC were back transformed in Table 3. The results of the linear regression per SD of ln HTGC show that in crude analyses and in age- and sex-adjusted models, levels of all coagulation factors were associated with ln HTGC. With further adjustment for demographic and lifestyle factors (model 3), associations of ln HTGC with coagulation factors did not substantially change. In multivariate models, one SD in ln HTGC was associated with higher levels of fibrinogen (6.3 mg/dL, 95% CI: 2.6, 9.9), factor VIII (3.2 IU/dL, 95% CI: 1.1, 5.4), factor IX (9.7 IU/dL, 95% CI: 8.5, 10.9) and factor XI (3.1 IU/dL, 95% CI: 1.9, 4.4). With additional adjustment for VAT and TBF (model 4), the associations between ln HTGC and levels of coagulation factors disappeared for factor VIII (1.6 IU/dL, 95% CI: -0.9, 4.2) and factor XI (1.2, 95% CI: -0.3, 2.7), or even became negative for fibrinogen (-5.0 mg/dL, 95% CI: -9.2, -0.7). However, the association between ln HTGC and factor IX levels, albeit attenuated, persisted (6.6 IU/dL, 95% CI: 5.1, 8.1).

Next, we examined the mean differences in coagulation factor levels for each quartile of ln HTGC in comparison with the first quartile used as the reference category (Table 3). Levels of coagulation factors increased dose-dependently across ln HTGC quartiles compared with the reference in crude analyses (model 1), and after adjustment for demographic and lifestyle factors (models 2 and 3). With additional adjustment for TBF and VAT (model 4), the associations between ln HTGC and levels of fibrinogen, factor VIII and factor XI across quartiles disappeared, whereas the association between ln HTGC and factor IX levels persisted, as did the dose-response relation.

Table 2. Association between body fat measures and hepatic triglyceride content in 1946 participants from the NEO study

	Difference in hepatic triglyceride content (%) (95% CI)[a] per SD of:					
	BMI (SD = 3.9 kg/m²)	**TBF (SD = 8.3%)**	**WC (SD = 12.6 cm)**	**VAT (SD = 54.5 cm²)**	**SAT (SD = 96.5 cm²)**	**VAT/SAT (SD = 0.61)**
Model 1	0.54 (0.49, 0.58)	0.18 (0.12, 0.24)	0.61 (0.57, 0.66)	0.65 (0.60, 0.69)	0.34 (0.29, 0.40)	0.48 (0.43, 0.53)
Model 2	0.50 (0.46, 0.55)	0.73 (0.66, 0.80)	0.61 (0.55, 0.66)	0.62 (0.57, 0.68)	0.46 (0.41, 0.51)	0.49 (0.41, 0.57)
Model 3	0.48 (0.44, 0.53)	0.69 (0.62, 0.75)	0.58 (0.52, 0.63)	0.59 (0.54, 0.64)	0.43 (0.38, 0.48)	0.46 (0.38, 0.54)

Results were based on analyses weighted towards a normal body mass index distribution. Data were missed for some participants in some subgroups.
Hepatic triglyceride content and the ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue were natural log-transformed.
BMI, body mass index; CI, Confidence Interval; SAT, abdominal subcutaneous adipose tissue; TBF, total body fat; VAT, visceral adipose tissue; VAT/SAT, ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue; WC, waist circumference.
[a]Beta coefficients (95% CI) from linear regression per weighted SD in BMI, TBF, WC, VAT, abdominal SAT, and VAT/SAT ratio.
Model 1: crude association.
Model 2: adjustment for age and sex.
Model 3: model 2 + adjustment for ethnicity, education level, alcohol intake, physical activity, tobacco smoking, estrogen use and menopause status.

Table 3. Association between hepatic triglyceride content and coagulation factor levels in 1946 participants from the NEO study

	Continuous scale	Reference (mean levels)	Mean difference (95% CI)[b] compared with the reference category		
	Difference in coagulation factor levels (95% CI)[a] per SD of HTGC (2.92%)[c]	**HTGC Quartile 1 <1.34%[c] (25%)**	**HTGC Quartile 2 1.34%-2.66%[c] (25%)**	**HTGC Quartile 3 2.66%-6.27%[c] (25%)**	**HTGC Quartile 4 ≥6.27%[c] (25%)**
Fibrinogen (mg/dL)					
Model 1	5.1 (2.0, 8.3)	281	7.3 (-3.5, 18.2)	11.1 (0.6, 21.6)	11.9 (2.2, 21.6)
Model 2	6.1 (2.8, 9.5)		7.0 (-4.2, 18.1)	12.7 (0.6, 24.9)	15.0 (3.8, 26.2)
Model 3	6.3 (2.6, 9.9)		6.3 (-5.3, 17.8)	13.7 (1.0, 26.3)	14.4 (1.8, 26.9)
Model 4	-5.0 (-9.2, -0.7)		-5.5 (-17.6, 6.7)	-10.3 (-25.0, 4.3)	-14.1 (-31.0, 2.2)
Factor VIII (IU/dL)					
Model 1	2.9 (0.9, 5.0)	119	2.0 (-4.6, 8.6)	2.7 (-3.5, 8.8)	7.1 (1.1, 13.1)
Model 2	3.0 (0.9, 5.0)		1.2 (-5.4, 7.9)	2.0 (-4.5, 8.6)	6.2 (0.1, 12.3)
Model 3	3.2 (1.1, 5.4)		1.4 (-5.4, 8.2)	2.5 (-4.1, 9.1)	6.6 (0.4, 12.8)
Model 4	1.6 (-0.9, 4.2)		-1.2 (-8.3, 5.9)	-1.9 (-9.2, 5.3)	3.2 (-5.1, 11.5)
Factor IX (IU/dL)					
Model 1	9.7 (8.5, 10.8)	103	9.8 (6.8, 12.8)	17.7 (14.4, 21.1)	26.2 (22.9, 29.5)
Model 2	9.9 (8.7, 11.0)		9.7 (6.6, 12.7)	18.6 (14.8, 22.3)	26.3 (23.0, 29.6)
Model 3	9.7 (8.5, 10.9)		9.8 (6.7, 12.9)	18.4 (14.8, 22.1)	26.1 (22.4, 29.8)
Model 4	6.6 (5.1, 8.1)		6.6 (3.6, 9.6)	11.7 (7.8, 15.6)	19.0 (13.8, 24.1)

Table 3. (continued)

	Continuous scale	Reference (mean levels)	Mean difference (95% CI)[b] compared with the reference category		
	Difference in coagulation factor levels (95% CI)[a] per SD of HTGC (2.92%)[c]	**HTGC Quartile 1 <1.34%[c] (25%)**	**HTGC Quartile 2 1.34%-2.66%[c] (25%)**	**HTGC Quartile 3 2.66%-6.27%[c] (25%)**	**HTGC Quartile 4 ≥6.27%[c] (25%)**
Factor XI (IU/dL)					
Model 1	2.0 (0.8, 3.1)	112	3.5 (-0.4, 7.4)	5.0 (1.2, 8.7)	5.8 (2.5, 9.2)
Model 2	3.3 (2.2, 4.5)		4.8 (1.0, 8.5)	7.7 (3.7, 11.7)	8.9 (5.3, 12.5)
Model 3	3.1 (1.9, 4.4)		4.2 (0.2, 8.1)	7.2 (2.9, 11.6)	8.4 (4.4, 12.5)
Model 4	1.2 (-0.3, 2.7)		2.4 (-1.8, 6.5)	2.1 (-2.7, 6.9)	4.9 (-0.3, 10.2)

Results were based on analyses weighted towards a normal body mass index distribution. Data were missed for some participants in some subgroups.

CI, confidence interval; HTGC, hepatic triglyceride content; SD, standard deviation.

[a] Beta coefficients (95% CI) from linear regression per weighted SD in natural log-transformed HTGC.

[b] Beta coefficients (95% CI) obtained by linear regression in each weighted quartile of natural log-transformed HTGC compared with the lowest quartile (reference category).

[c] Values of SD and quartiles of HTGC were back transformed for interpretation.

Model 1: crude association.

Model 2: adjustment for age and sex.

Model 3: model 2 + adjustment for ethnicity, education level, alcohol intake, physical activity, tobacco smoking, estrogen use and menopause status.

Model 4: model 3 + adjustment for visceral adipose tissue and total body fat.

Table 4 presents mean differences in coagulation factor levels for each quartile of ln HTGC in comparison with the first quartile stratified by sex. For reporting and interpretation, values of ln HTGC were also back transformed in Table 4. As in overall analysis, upon adjustment for TBF and VAT, no associations between ln HTGC and levels of fibrinogen or factor VIII were observed in both men and women. Compared with the reference category, higher quartiles of ln HTGC were associated with higher levels of factor IX in a dose-response fashion and to a similar extent in men and women, also after adjustment for VAT and TBF. Interestingly, ln HTGC was consistently associated with factor XI levels in women, even with further adjustment for TBF and VAT, but not in men, in whom the associations were weak or absent across quartiles and regression models. After excluding participants with alcohol consumption ≥20g/day, results were similar for overall (Table S4) and subgroup analyses stratified by sex (Table S5).

DISCUSSION

In this large population-based cross-sectional study, HTGC was associated with levels of various coagulation factors (i.e., fibrinogen, factor VIII, factor IX or factor XI) in a dose-response fashion, even after adjustment for several demographic and lifestyle potential confounding factors. However, with further adjustment for total body and visceral fat, the associations between HTGC and levels of fibrinogen, factor VIII and factor XI disappeared, whereas the associations between HTGC and factor IX levels, albeit attenuated, persisted, as did the dose-response relation. This observation could be relevant, as high levels of factor IX related to liver fat content have the potential to be a critical pathway by which obesity increases the risk of venous thrombosis. Indeed, there is substantial body of evidence that factor IX, a vitamin K-dependent factor (VKDF), plays a pivotal role in thrombin generation [40,41]. Furthermore, high levels of factor IX have been associated with an increased risk of venous thrombosis in epidemiological studies [42-44].

To the best of our knowledge, this is the first study to show that HTGC and factor IX levels are associated in a dose-response fashion, also after adjustment for several potential confounding factors, including total body and visceral fat. Interestingly, our finding on the association of hepatic triglycerides with factor IX levels seems to be in line with previous studies on principal component analysis, in which serum triglycerides clustered together with factor IX and other procoagulant VKDFs, i.e., factors II, VII, and X [45,46]. Recently, we have assessed the interrelation between levels of several hemostatic factors and lipids in 2874 population controls from the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study. We found that serum triglycerides clustered together with all VKDFs, including the procoagulant and anticoagulant factors [47]. Taken together,

Table 4. Association between hepatic triglyceride content and coagulation factor levels in 1946 participants from the NEO study according to sex

	Reference (mean levels)	Mean difference (95% CI)[a] compared with the reference category		
	HTGC Quartile 1 <1.34%[b]	**HTGC Quartile 2 1.34%-2.66%[b]**	**HTGC Quartile 3 2.66%-6.27%[b]**	**HTGC Quartile 4 ≥6.27%[b]**
Fibrinogen (mg/dL)				
Model 1				
Men	276	7.3 (-15.1, 29.7)	9.6 (-11.6, 30.7)	7.3 (-13.1, 27.6)
Women	283	9.5 (-3.3, 22.7)	18.8 (5.1, 32.6)	26.2 (13.2, 39.2)
Model 2				
Men	276	7.1 (-15.3, 29.4)	7.2 (-13.6, 28.1)	5.7 (-14.3, 25.8)
Women	283	6.6 (-6.1, 19.3)	16.8 (1.6, 32.1)	21.4 (8.2, 34.6)
Model 3				
Men	276	5.3 (-19.7, 30.3)	9.8 (-12.8, 32.5)	3.5 (-18.7, 25.8)
Women	283	6.6 (-5.7, 18.8)	17.2 (3.0, 31.3)	25.5 (12.3, 38.7)
Model 4				
Men	276	-4.2 (-30.3, 22.0)	-10.6 (-36.2, 15.1)	-16.0 (-44.4, 12.4)
Women	283	-6.1 (-19.0, 6.7)	-10.4 (-25.7, 4.9)	-11.2 (-29.2, 6.8)
Factor VIII (IU/dL)				
Model 1				
Men	120	-1.2 (-10.9, 8.5)	-2.1 (-10.8, 6.6)	4.5 (-4.1, 13.2)
Women	119	4.0 (-4.7, 12.7)	8.4 (-0.2, 17.0)	10.0 (1.8, 18.3)
Model 2				
Men	120	-1.4 (-11.0, 8.3)	-3.9 (-12.6, 4.8)	3.2 (-5.4, 11.7)
Women	119	2.3 (-6.6, 11.2)	6.2 (-3.4, 15.8)	9.0 (0.1, 17.9)
Model 3				
Men	120	-1.2 (-10.9, 8.4)	-3.6 (-12.0, 4.8)	3.3 (-5.1, 11.7)
Women	119	2.4 (-6.9, 11.6)	6.8 (-2.4, 16.0)	9.2 (0.6, 17.7)
Model 4				
Men	120	-2.7 (-12.5, 7.2)	-4.1 (-13.6, 5.3)	3.0 (-8.3, 14.2)
Women	119	-0.8 (-11.0, 9.3)	-2.1 (-12.2, 8.0)	2.0 (-10.3, 14.2)
Factor IX (IU/dL)				
Model 1				
Men	103	9.1 (4.0, 14.2)	14.0 (8.9, 19.1)	24.1 (18.7, 29.4)
Women	102	9.9 (6.1, 13.8)	22.1 (17.1, 27.1)	28.6 (24.5, 32.8)
Model 2				
Men	103	9.0 (4.0, 14.1)	13.4 (8.4, 18.3)	23.5 (18.2, 28.7)
Women	102	10.2 (6.4, 14.0)	22.4 (17.0, 27.8)	28.8 (24.6, 33.0)
Model 3				
Men	103	8.6 (3.2, 13.9)	11.8 (6.9, 16.7)	20.6 (14.4, 26.9)
Women	102	10.3 (6.3, 14.2)	22.7 (17.8, 27.6)	29.8 (26.0, 33.7)
Model 4				
Men	103	6.8 (1.5, 12.0)	8.0 (2.6, 13.4)	16.3 (8.1, 24.6)
Women	102	5.9 (2.1, 9.7)	12.7 (7.8, 17.5)	19.0 (13.2, 24.9)

Table 4. (continued)

	Reference (mean levels)	Mean difference (95% CI)[a] compared with the reference category		
	HTGC Quartile 1 <1.34%[b]	**HTGC Quartile 2 1.34%-2.66%[b]**	**HTGC Quartile 3 2.66%-6.27%[b]**	**HTGC Quartile 4 ≥6.27%[b]**
Factor XI (IU/dL)				
Model 1				
Men	109	1.1 (-5.7, 7.9)	2.4 (-3.5, 8.2)	4.6 (-0.9, 10.1)
Women	113	6.6 (2.1, 11.2)	12.2 (7.1, 17.3)	12.1 (7.4, 16.8)
Model 2				
Men	109	1.2 (-5.4, 7.9)	2.5 (-3.4, 8.4)	5.0 (-0.5, 10.5)
Women	113	5.8 (1.2, 10.3)	11.0 (5.5, 16.5)	11.3 (6.5, 16.2)
Model 3				
Men	109	1.0 (-6.5, 8.4)	1.0 (-5.6, 7.5)	2.9 (-3.2, 9.1)
Women	113	4.8 (0.2, 9.5)	11.5 (6.0, 17.0)	13.1 (8.1, 18.2)
Model 4				
Men	109	0.1 (-7.8, 7.9)	-3.7 (-10.9, 3.6)	-1.2 (-8.3, 5.9)
Women	113	2.1 (-2.6, 6.8)	6.3 (0.2, 12.5)	11.5 (4.2, 18.8)

Results were based on weighted analyses towards a normal body mass index distribution. Data were missed for some participants in some subgroups. HTGC, hepatic triglyceride content; CI, confidence interval.

[a] Beta coefficients (95% CI) obtained by linear regression in each weighted quartile of natural log-transformed HTGC compared with the lowest quartile (reference category).

[b] Values of HTCG quartiles were back transformed for interpretation.

Model 1: crude association. Model 2: adjustment for age. Model 3: model 2 + adjustment for ethnicity, education level, alcohol intake, physical activity, tobacco smoking, estrogen use (women only) and menopause status (women only). Model 4: model 3 + adjustment for visceral adipose tissue and total body fat.

results from these studies support the existence of an interrelation between serum triglycerides and VKDFs.

The mechanism underlying the relationship between factor IX levels and HTGC is as yet unknown. Still, since the liver is the main site of production of coagulation factors [48], it is biologically plausible to speculate that the observed association could be related to pathways involved in the synthesis of factor IX. Recently, gene expression of various coagulation factors has been investigated in NASH [49]. Compared with healthy individuals, NASH patients had increased hepatic triglyceride levels but reduced hepatic mRNA levels of factor IX and of several other coagulation factors. Although our results on the positive association between HTGC and plasma activity of factor IX do not seem to be in line with the above mentioned findings [49], it is important to address that gene expression of coagulation factors is not known in detail in NAFLD, and across the different stages of the disease (i.e., from simple steatosis

to NASH and cirrhosis). Alternatively, other mechanisms involved in the synthesis of factor IX could also influence its plasma activity without affecting its transcript levels, such as post-transcriptional or post-translational changes. For instance, Cleuren *et al*. [50] have recently demonstrated an increase in plasma activity of several coagulation factors (i.e., fibrinogen, and factors II, VII, VIII, IX, XI and XII) in mice kept on high fat diet for 14 days. However, with the exception of factor XI, the increase in plasma activity was not paralleled by changes in gene expression of these coagulation factors in the liver [50].

Here, one may also consider that the association between HTGC and factor IX could be explained by common mechanisms regulating both liver fat content and factor IX levels. Vanschoonbeek *et al*. [51] have shown in a murine model of type III hyperlipidemia that mice kept on fish oil diet (n-3 polyunsaturated fatty acids [n-3 PUFAs]) for 21 days had a reduction in plasma triglyceride levels, thrombin generation, and activity of VKDFs, but not in VKDF mRNA levels, which remained unchanged. Analysis of mouse livers showed that n-3 PUFA was associated with upregulation of genes related to lipid degradation, and downregulation of genes related to lipid synthesis and of γ-glutamyl carboxylase [51]. The latter gene encodes the enzyme responsible for the γ-carboxylation of VKDFs, which is a fundamental post-translational step for the activity of these factors in blood coagulation [52]. Consistent with the murine model study [51] and with the reduction in triglyceride levels by n-3 PUFA in clinical studies [53], n-3 PUFA supplementation has been suggested to decrease liver fat content in humans [54]. Furthermore, some observational studies have found an inverse association between fish/n-3 PUFA intake or n-3 PUFA blood levels and risk of venous thrombosis [55,56], including recurrent events [57]. The mechanism behind this inverse association is not fully understood, and may include downregulation of the activity of procoagulant VKDFs, such as factor IX. Taken together, there might be common mechanism(s) regulating the metabolism of lipids and the activity of VKDFs in the hepatocytes, which could explain, at least in part, our results on the strong association between HTGC and factor IX plasma activity. Therapeutic and lifestyle strategies targeting possible common mechanisms might decrease not only liver fat content and factor IX activity, but also the risk of venous thrombosis. Hence, further studies aimed to unravel the pathophysiology behind the association between HTGC and factor IX levels are important, both from a mechanistic and a clinical viewpoint.

In the present study, the associations between HTGC and levels of fibrinogen and factor VIII disappeared upon adjustment for TBF and VAT, thereby suggesting a close link of adipose tissue with both factors. Our results confirm previous studies, in which body fat measures were closely related to levels of fibrinogen [6,23,58] and factor VIII [23,59]. Adipose tissue may influence the regulation of fibrinogen and factor VIII levels possibly through the secretion of bioactive factors, such as pro-

inflammatory cytokines [60]. Fibrinogen, produced by hepatocytes [61], and factor VIII, produced in the liver by endothelial cells [62,63], are well-known for acting as acute-phase proteins [61,64]. For instance, interleukin-6 (IL-6), a pro-inflammatory cytokine secreted by adipose tissue [60], up-regulates the expression of both factors at the transcriptional level [61,64]. Furthermore, fibrinogen and factor VIII levels have been shown to cluster together with C-reactive protein (CRP), an inflammatory marker, rather than markers of procoagulant activity [45,47]. On the whole, based on our results, total body and visceral fat appear to largely explain the associations of HTGC with fibrinogen and factor VIII levels, and to a lesser extent, with factor XI levels. Notably, the association between HTGC and factor IX levels also appears to be partly explained by overall and visceral fat given the attenuation of this association upon adjustment for TBF and VAT. It is noteworthy that similarly to fibrinogen and factor VIII, factor IX levels were associated with IL-6 levels [65] and clustered together with CRP [45,47]. However, it remains open whether and how bioactive factors related to fat-cell biosynthesis mediate the effect of total body and visceral fat on fibrinogen and factors VIII, IX and XI levels, and further investigation on this topic is warranted.

In subgroup analyses stratified by sex, results were similar to the overall analysis, with the exception of factor XI, which levels were consistently associated with HTGC in women, in a dose-response fashion, even after adjustment for total body and visceral fat. As far as we know, the effect of sex on the association between HTGC and coagulation factors has not been studied before, and whether there is a biological reason behind the observed sex difference in factor XI levels remains to be clarified.

Strengths of the present study include the availability of ^{1}H MRS to quantify HTGC in combination with MRI to quantify VAT, and a more accurate measure of TBF (i.e., bioelectrical impedance analysis) than BMI. This enabled us to adjust all analyses for total body and visceral fat. Moreover, the strong associations of TBF and VAT with HTGC observed in this study underscores the need for taking into account both measures when studying specific effects of liver fat. Further strengths are the large study population and the information on multiple potential confounding factors. Because of the large sample size, we were able to categorize HTGC into quartiles, and assess a dose-response relation between HTGC and levels of coagulation factors; to adjust for several potential confounding factors; and to investigate possible sex differences.

Limitations of this study should also be addressed. First, the observational, cross-sectional nature of the present study precludes causal inferences related to our results. Second, as in all observational designs, we cannot exclude the presence of residual confounding due to unknown or unmeasured confounding factors. Third, since for obvious ethical reasons we could not perform liver biopsies, we were unable to determine whether levels of coagulation factors differ across the histological

stages of NAFLD. Fourth, among the VKDFs, we evaluated factor IX only. Whether HTGC is related to other VKDFs as well, either procoagulant (factors II, VII and X) or anticoagulant (protein C and protein S) factors, and whether the relationship between HTGC and VKDFs results in a hypercoagulable state may deserve further investigation. Finally, our study population consisted primarily of white individuals aged between 45-65 years, and our results may therefore not be generalizable to other ethnic or age groups.

In conclusion, HTGC was associated with levels of fibrinogen, and factors VIII, IX and XI, of which factor IX remained associated with HTGC after adjustment for TBF and VAT. Our results shed more light on the relation between obesity and venous thrombosis risk, including the potential that HTGC contributes to venous thrombosis risk beyond total body and visceral fat through factor IX levels.

REFERENCES

1. Braekkan SK, Siegerink B, Lijfering WM, Hansen JB, Cannegieter SC, Rosendaal FR. Role of obesity in the etiology of deep vein thrombosis and pulmonary embolism: current epidemiological insights. Semin Thromb Hemost 2013; 39: 533-40.
2. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. Science 2011; 332: 1519-23.
3. Targher G, Byrne CD. Diagnosis and management of nonalcoholic fatty liver disease and its hemostatic/thrombotic and vascular complications. Semin Thromb Hemost 2013; 39: 214-28.
4. Di Minno MN, Tufano A, Rusolillo A, Di Minno G, Tarantino G. High prevalence of nonalcoholic fatty liver in patients with idiopathic venous thromboembolism. World J Gastroenterol 2010; 16: 6119-22.
5. Cigolini M, Targher G, Agostino G, Tonoli M, Muggeo M, De Sandre G. Liver steatosis and its relation to plasma haemostatic factors in apparently healthy men-role of the metabolic syndrome. Thromb Haemost 1996; 76: 69-73.
6. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. Diabet Med 2005; 22: 1354-58.
7. Yener S, Akarsu M, Demir T, Akinci B, Sagol O, Bayraktar F, Ozcan MA, Tankurt E, Yesil S. Plasminogen activator inhibitor-1 and thrombin activatable fibrinolysis inhibitor levels in non-alcoholic steatohepatitis. J Endocrinol Invest 2007; 30: 810-19.
8. Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, Königsrainer A, Maier KP, Bischoff SC, Bergheim I. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. J Nutr 2008; 138: 1452-55.
9. de Larrañaga G, Wingeyer SP, Graffigna M, Belli S, Bendezú K, Alvarez S, Levalle O, Fainboim H. Plasma plasminogen activator inhibitor-1 levels and nonalcoholic fatty liver in individuals with features of metabolic syndrome. Clin Appl Thromb Hemost 2008; 14: 319-24.
10. Targher G, Bertolini L, Rodella S, Lippi G, Franchini M, Zoppini G, Muggeo M, Day CP. NASH predicts plasma inflammatory biomarkers independently of visceral fat in men. Obesity 2008; 16: 1394-99.
11. Barbato A, Iacone R, Tarantino G, Russo O, Sorrentino P, Avallone S, Galletti F, Farinaro E, Della Valle E, Strazzullo P; Olivetti Heart Study Research Group. Relationships of PAI-1 levels to central obesity and liver steatosis in a sample of adult male population in southern Italy. Intern Emerg Med 2009; 4: 315-23.
12. Kargili A, Cipil H, Karakurt F, Kasapoglu B, Koca C, Aydin M, Uysal S, Ikizek M, Uz E, Balcik OS, Kosar A. Hemostatic alterations in fatty liver disease. Blood Coagul Fibrinolysis 2010; 21: 325-27.
13. Sookoian S, Castaño GO, Burgueño AL, Rosselli MS, Gianotti TF, Mallardi P, Martino JS, Pirola CJ. Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. Atherosclerosis 2010; 209: 585-91.

14. Bilgir O, Bilgir F, Bozkaya G, Calan M. Changes in the levels of endothelium-derived coagulation parameters in nonalcoholic fatty liver disease. Blood Coagul Fibrinolysis 2014; 25: 151-55.

15. Verrijken A, Francque S, Mertens I, Prawitt J, Caron S, Hubens G, Van Marck E, Staels B, Michielsen P, Van Gaal L. Prothrombotic factors in histologically proven nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology 2014; 59: 121-29.

16. Chang ML, Hsu CM, Tseng JH, Tsou YK, Chen SC, Shiau SS, Yeh CT, Chiu CT. Plasminogen activator inhibitor-1 is independently associated with non-alcoholic fatty liver disease whereas leptin and adiponectin vary between genders. J Gastroenterol Hepatol 2015; 30: 329-36.

17. Potze W, Siddiqui MS, Boyett SL, Adelmeijer J, Daita K, Sanyal AJ, Lisman T. Preserved hemostatic status in patients with non-alcoholic fatty liver disease. J Hepatol 2016; 65: 980-87.

18. Kotronen A, Joutsi-Korhonen L, Sevastianova K, Bergholm R, Hakkarainen A, Pietiläinen KH, Lundbom N, Rissanen A, Lassila R, Yki-Järvinen H. Increased coagulation factor VIII, IX, XI and XII activities in non-alcoholic fatty liver disease. Liver Int 2011; 31: 176-183.

19. Tripodi A, Fracanzani AL, Primignani M, Chantarangkul V, Clerici M, Mannucci PM, Peyvandi F, Bertelli C, Valenti L, Fargion S. Procoagulant imbalance in patients with non-alcoholic fatty liver disease. J Hepatol 2014; 61: 148-54.

20. Rosendaal FR. Venous thrombosis: the role of genes, environment, and behavior. Hematology Am Soc Hematol Educ Program 2005:1-12.

21. Speliotes EK, Massaro JM, Hoffmann U, Vasan RS, Meigs JB, Sahani DV, Hirschhorn JN, O'Donnell CJ, Fox CS. Fatty liver is associated with dyslipidemia and dysglycemia independent of visceral fat: the Framingham Heart Study. Hepatology 2010; 51:1979-87.

22. Yaskolka Meir A, Tene L, Cohen N, Shelef I, Schwarzfuchs D, Gepner Y, Zelicha H, Rein M, Bril N, Serfaty D, Kenigsbuch S, Chassidim Y, Sarusy B, Dicker D, Thiery J, Ceglarek U, Stumvoll M, Blüher M, Stampfer MJ, Rudich A, Shai I. Intrahepatic fat, abdominal adipose tissues, and metabolic state: magnetic resonance imaging study. Diabetes Metab Res Rev 2017; 33(5).

23. Cigolini M, Targher G, Bergamo Andreis IA, Tonoli M, Agostino G, De Sandre G. Visceral fat accumulation and its relation to plasma hemostatic factors in healthy men. Arterioscler Thromb Vasc Biol 1996; 16: 368-74.

24. Seidell JC, Bouchard C. Visceral fat in relation to health: is it a major culprit or simply an innocent bystander? Int J Obes Relat Metab Disord 1997; 21: 626-31.

25. Martin AD, Janssens V, Caboor D, Clarys JP, Marfell-Jones MJ. Relationships between visceral, trunk and whole-body adipose tissue weights by cadaver dissection. Ann Hum Biol 2003; 30: 668-77.

26. Gast KB, den Heijer M, Smit JW, Widya RL, Lamb HJ, de Roos A, Jukema JW, Rosendaal FR, de Mutsert R; NEO study group. Individual contributions of visceral fat and total body fat to subclinical atherosclerosis: The NEO study. Atherosclerosis 2015; 241: 547-54.

27. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, Hobbs HH, Dobbins RL. Magnetic resonance spectroscopy to measure hepatic triglyceride content:

prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab 2005; 288: E462-E468.

28. Adams LA, Lindor KD. Nonalcoholic fatty liver disease. Ann Epidemiol 2007; 17: 863-69.
29. Zelber-Sagi S, Godos J, Salomone F. Lifestyle changes for the treatment of nonalcoholic fatty liver disease: a review of observational studies and intervention trials. Therap Adv Gastroenterol 2016; 9: 392-407.
30. Nguyen MC, Stewart RB, Banerji MA, Gordon DH, Kral JG. Relationships between tamoxifen use, liver fat and body fat distribution in women with breast cancer. Int J Obes Relat Metab Disord 2001; 25: 296-98.
31. Woodward M, Lowe GD, Rumley A, Tunstall-Pedoe H, Philippou H, Lane DA, Morrison CE. Epidemiology of coagulation factors, inhibitors and activation markers: The Third Glasgow MONICA Survey. II. Relationships to cardiovascular risk factors and prevalent cardiovascular disease. Br J Haematol 1997; 97: 785-97.
32. Tchaikovski SN, Rosing J. Mechanisms of estrogen-induced venous thromboembolism. Thromb Res 2010; 126: 5-11.
33. Tchernof A, Després JP. Pathophysiology of human visceral obesity: an update. Physiol Rev 2013; 93: 359-404.
34. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology 2004; 40: 1387-95.
35. Ulbrich EJ, Fischer MA, Manoliu A, Marcon M, Luechinger R, Nanz D, Reiner CS. Age- and Gender Dependent Liver Fat Content in a Healthy Normal BMI Population as Quantified by Fat-Water Separating DIXON MR Imaging. PLoS One 2015; 10: e0141691.
36. Roach RE, Cannegieter SC, Lijfering WM. Differential risks in men and women for first and recurrent venous thrombosis: the role of genes and environment. J Thromb Haemost 2014; 12: 1593-1600.
37. de Mutsert R, den Heijer M, Rabelink TJ, Smit JW, Romijn JA, Jukema JW, de Roos A, Cobbaert CM, Kloppenburg M, le Cessie S, Middeldorp S, Rosendaal FR. The Netherlands Epidemiology of Obesity (NEO) study: study design and data collection. Eur J Epidemiol 2013; 28: 513-23.
38. Widya RL, de Mutsert R, den Heijer M, le Cessie S, Rosendaal FR, Jukema JW, Smit JW, de Roos A, Lamb HJ; NEO Study Group. Association between Hepatic Triglyceride Content and Left Ventricular Diastolic Function in a Population-based Cohort: The Netherlands Epidemiology of Obesity Study. Radiology 2016; 279: 443-50.
39. Clauss A. Rapid physiological coagulation method in determination of fibrinogen [in German]. Acta Haematol 1957; 17: 237-46.
40. Mann KG, Brummel-Ziedins K, Orfeo T, Butenas S.Models of blood coagulation. Blood Cells Mol Dis 2006; 36: 108-17.

41. Eikelboom JW, Zelenkofske SL, Rusconi CP. Coagulation factor IXa as a target for treatment and prophylaxis of venous thromboembolism. Arterioscler Thromb Vasc Biol 2010; 30: 382-387.

42. van Hylckama Vlieg A, van der Linden IK, Bertina RM, Rosendaal FR. High levels of factor IX increase the risk of venous thrombosis. Blood 2000; 95: 3678-82.

43. Lowe G, Woodward M, Vessey M, Rumley A, Gough P, Daly E. Thrombotic variables and risk of idiopathic venous thromboembolism in women aged 45-64 years. Relationships to hormone replacement therapy. Thromb Haemost 2000; 83: 530-35.

44. Campello E, Spiezia L, Bulato C, Gavasso S, Woodhams B, Simioni P. Factor IX activity/antigen ratio and the risk of first unprovoked venous thromboembolism. Thromb Haemost 2013; 109: 755-56.

45. Sakkinen PA, Wahl P, Cushman M, Lewis MR, Tracy RP. Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome. Am J Epidemiol 2000; 152: 897-907.

46. Vanschoonbeek K, Feijge MA, Saris WH, de Maat MP, Heemskerk JW. Plasma triacylglycerol and coagulation factor concentrations predict the anticoagulant effect of dietary fish oil in overweight subjects. J Nutr 2007; 137: 7-13.

47. Morelli VM, Li-Gao R, Lijfering WM, Rosendaal FR, Cannegieter SC, van Hylckama Vlieg A. Interrelation between levels of hemostatic factors, lipids and c-reactive protein in population controls. Res Pract Thromb Haemost 2017; 1 (Suppl. 1): 388-9.

48. Brummel-Ziedins K, Orfeo T, Jenny NS, et al. Blood coagulation and fibrinolysis. In: Greer JP, Foerster J, Rodgers GM, Paraskevas F, Glader B, Arber DA, Means RT, eds. Wintrobe's Clinical Hematology. Philadelphia: Lippincott Williams & Wilkins; 2009: 528-619.

49. Xu Y, Zalzala M, Xu J, Li Y, Yin L, Zhang Y. A metabolic stress-inducible miR-34a-HNF4α pathway regulates lipid and lipoprotein metabolism. Nat Commun 2015; 6: 7466.

50. Cleuren AC, Blankevoort VT, van Diepen JA, Verhoef D, Voshol PJ, Reitsma PH, van Vlijmen BJ. Changes in Dietary Fat Content Rapidly Alters the Mouse Plasma Coagulation Profile without Affecting Relative Transcript Levels of Coagulation Factors. PLoS One 2015; 10: e0131859.

51. Vanschoonbeek K, Wouters K, van der Meijden PE, van Gorp PJ, Feijge MA, Herfs M, Schurgers LJ, Hofker MH, de Maat MP, Heemskerk JW. Anticoagulant effect of dietary fish oil in hyperlipidemia: a study of hepatic gene expression in APOE2 knock-in mice. Arterioscler Thromb Vasc Biol 2008; 28: 2023-29.

52. Oldenburg J, Watzka M, Rost S, Muller CR. VKORC1: molecular target of coumarins. J Thromb Haemost 2007; 5 (Suppl. 1):1-6.

53. Leslie MA, Cohen DJ, Liddle DM, Robinson LE, Ma DW. A review of the effect of omega-3 polyunsaturated fatty acids on blood triacylglycerol levels in normolipidemic and borderline hyperlipidemic individuals. Lipids Health Dis 2015; 14: 53.

54. Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol 2012; 56: 944-51.

55. Steffen LM, Folsom AR, Cushman M, Jacobs DR Jr, Rosamond WD. Greater fish, fruit, and vegetable intakes are related to lower incidence of venous thromboembolism: the Longitudinal Investigation of Thromboembolism Etiology. Circulation 2007; 115: 188-95.

56. Hansen-Krone IJ, Enga KF, Südduth-Klinger JM, Mathiesen EB, Njølstad I, Wilsgaard T, Watkins S, Brækkan SK, Hansen JB. High fish plus fish oil intake is associated with slightly reduced risk of venous thromboembolism: the Tromsø Study. J Nutr 2014; 144: 861-67.

57. Reiner MF, Stivala S, Limacher A, Bonetti NR, Méan M, Egloff M, Rodondi N, Aujesky D, von Schacky C, Lüscher TF, Camici GG, Beer JH. Omega-3 fatty acids predict recurrent venous thromboembolism or total mortality in elderly patients with acute venous thromboembolism. J Thromb Haemost 2017; 15: 47-56.

58. Ditschuneit HH, Flechtner-Mors M, Adler G. Fibrinogen in obesity before and after weight reduction. Obes Res 1995; 3: 43-48.

59. Abdollahi M, Cushman M, Rosendaal FR. Obesity: risk of venous thrombosis and the interaction with coagulation factor levels and oral contraceptive use. Thromb Haemost 2003; 89: 493-48.

60. Blüher M, Mantzoros CS. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. Metabolism 2015; 64: 131-45.

61. Fish RJ, Neerman-Arbez M. Fibrinogen gene regulation. Thromb Haemost 2012; 108: 419-26.

62. Shahani T, Covens K, Lavend'homme R, et al. Human liver sinusoidal endothelial cells but not hepatocytes contain factor VIII. J Thromb Haemost 2014; 12: 36-42.

63. Fahs SA, Hille MT, Shi Q, Weiler H, Montgomery RR. A conditional knockout mouse model reveals endothelial cells as the principal and possibly exclusive source of plasma factor VIII. Blood 2014; 123: 3706-13.

64. Kerr R, Stirling D, Ludlam CA. Interleukin 6 and haemostasis. Br J Haematol 2001; 115: 3-12.

65. Wannamethee SG, Whincup PH, Rumley A, Lowe GDO. Inter-relationships of interleukin-6, cardiovascular risk factors and the metabolic syndrome among older men. J Thromb Haemost 2007; 5: 1637-43.

SUPPLEMENTAL MATERIAL

Supplementary Table 1. Baseline characteristics of 1946 participants from the NEO study according to sex

Characteristics	Men (n = 1017)	Women (n = 929)
Demographic and lifestyle factors		
Age (years)	56 (50, 61)	55 (51, 60)
Ethnicity (% whites)	96	96
Education level (% high)[a]	51	43
Alcohol consumption (g/day)	16.8 (5.2, 27.9)	7.7 (1.6, 14.4)
Alcohol consumption (%)		
<10g/day	36	62
10-20g/day	22	19
20-40g/day	28	16
≥40g/day	14	3
Physical activity (MET-hours per week)	31.0 (15.0, 52.8)	29.5 (16.5, 49.5)
Tobacco smoking (% current)	15	13
Estrogen (% current use)	NA	10
Menopause status (% postmenopausal)	NA	81
Measures of adiposity		
BMI (kg/m^2)	26.6 (3.4)	25.3 (4.3)
Total body fat (%)	24.5 (5.5)	36.2 (6.4)
Waist circumference (cm)	97.4 (10.2)	85.4 (11.9)
VAT (cm^2)	113.0 (56.3)	66.2 (42.1)
SAT (cm^2)	205.6 (80.8)	256.8 (102.9)
VAT/SAT	0.52 (0.39, 0.69)	0.23 (0.17, 0.32)
Hepatic triglyceride content (%)	3.78 (1.98, 8.44)	1.82 (1.09, 4.65)
Coagulation factors		
Fibrinogen (mg/dL)	283 (55)	294 (54)
Factor VIII (IU/dL)	121 (31)	123 (34)
Factor IX (IU/dL)	118 (19)	115 (21)
Factor XI (IU/dL)	111 (18)	120 (20)
Transaminases		
ALT (U/L)	29.3 (13.1)	21.2 (8.1)
AST (U/L)	26.7 (9.3)	22.9 (6.3)

Results were based on analyses weighted towards a normal body mass index distribution. Data were missed for some participants in some subgroups. Data are shown as mean (± standard deviation), median (25th percentile -75th percentile) or percentage.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; MET, metabolic equivalents of task; NA, not applicable; SAT, abdominal subcutaneous adipose tissue; VAT, visceral adipose tissue; VAT/SAT, ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue; WC, waist circumference.

[a] High educational level (according to Dutch educational system): higher secondary education, higher vocational education, university, and PhD.

Supplementary Table 2. Baseline characteristics of 1326 participants from the NEO study with alcohol consumption <20g/day

Characteristics	All (n = 1326)	Men (n = 567)	Women (n = 759)
Demographic and lifestyle factors			
Age (years)	56 (50-60)	56 (50-61)	55 (51-60)
Ethnicity (% whites)	95	94	96
Education level (% high) [a]	42	50	37
Alcohol consumption (g/day)	4.8 (1.9-10.7)	7.0 (2.0-13.2)	4.1 (1.0-9.8)
Alcohol consumption (%)			
<10g/day	70	62	76
10-20g/day	30	38	24
Physical activity (MET-hours per week)	30.0 (15.3-51.2)	31.0 (14.7-54.0)	30.0 (16.1-49.0)
Tobacco smoking (% current)	10	11	10
Estrogen (% current use)	NA	NA	10
Menopause status (% postmenopausal)	NA	NA	81
Measures of adiposity			
BMI (kg/m^2)	25.8 (4.0)	26.3 (3.5)	25.4 (4.3)
Total body fat (%)	31.5 (8.5)	24.0 (5.6)	36.3 (6.3)
Waist circumference (cm)	90.0 (12.5)	96.6 (10.3)	85.7 (11.9)
VAT (cm^2)	82.0 (51.6)	106.6 (56.0)	66.4 (41.7)
SAT (cm^2)	238.3 (100.1)	202.6 (84.5)	260.9 (102.6)
VAT/SAT	0.31 (0.21-0.47)	0.50 (0.36-0.66)	0.23 (0.17-0.31)
Hepatic triglyceride content (%)	2.35 (1.22-5.8)	3.41 (1.82-7.75)	1.74 (1.08-4.54)
Coagulation factors			
Fibrinogen (mg/dL)	293 (55)	285 (56)	298 (54)
Factor VIII (IU/dL)	123 (32)	122 (30)	123 (34)
Factor IX (IU/dL)	115 (20)	116 (19)	114 (21)
Factor XI (IU/dL)	116 (20)	111 (18)	119 (20)
Transaminases			
ALT (U/L)	23.8 (10.2)	27.7 (11.9)	21.2 (8.0)
AST (U/L)	23.9 (6.6)	25.6 (6.6)	22.8 (6.4)

Results were based on weighted analyses towards a normal body mass index distribution. Data were missed for some participants in some subgroups. Data are shown as mean (± standard deviation), median (25th percentile -75th percentile) or percentage.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; MET, metabolic equivalents of task; NA, not applicable; SAT, abdominal subcutaneous adipose tissue; VAT, visceral adipose tissue; VAT/SAT, ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue; WC, waist circumference.

[a] High educational level (according to Dutch educational system): higher secondary education, higher vocational education, university, and PhD.

Supplementary Table 3. Association between body fat measures and hepatic triglyceride content in 1326 participants from the NEO study with alcohol consumption <20g/day

	Difference in hepatic triglyceride content (%) (95% CI)[a] per SD of:					
	BMI (SD = 4.0 kg/m²)	**TBF (SD = 8.5%)**	**WC (SD = 12.5 cm)**	**VAT (SD = 51.6 cm²)**	**SAT (SD = 100.1 cm²)**	**VAT/SAT (SD = 0.60)**
Model 1	0.55 (0.48, 0.59)	0.22 (0.15, 0.29)	0.61 (0.56, 0.66)	0.63 (0.57, 0.68)	0.36 (0.30, 0.43)	0.44 (0.37, 0.51)
Model 2	0.52 (0.47, 0.57)	0.76 (0.68, 0.84)	0.61 (0.55, 0.67)	0.61 (0.55, 0.67)	0.48 (0.42, 0.54)	0.44 (0.35, 0.54)
Model 3	0.50 (0.45, 0.56)	0.72 (0.64, 0.81)	0.58 (0.52, 0.65)	0.59 (0.54, 0.65)	0.45 (0.39, 0.52)	0.44 (0.35, 0.53)

Results were based on analyses weighted towards a normal body mass index distribution. Data were missed for some participants in some subgroups.

Hepatic triglyceride content and the ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue were log-transformed.

BMI, body mass index; CI, Confidence Interval; SAT, abdominal subcutaneous adipose tissue; TBF, total body fat; VAT, visceral adipose tissue; VAT/SAT, ratio of visceral adipose tissue and abdominal subcutaneous adipose tissue; WC, waist circumference.

[a]Beta coefficients (95% CI) from linear regression per weighted SD in BMI, TBF, WC, VAT, abdominal SAT, and VAT/SAT ratio.

Model 1: crude association.

Model 2: adjustment for age and sex.

Model 3: model 2 + adjustment for ethnicity, education level, alcohol intake, physical activity, tobacco smoking, estrogen use and menopause status.

Supplementary Table 4. Association between hepatic triglyceride content and coagulation factor levels in 1326 participants from the NEO study with alcohol consumption <20g/day

	Continuous scale	Reference (mean levels)	Mean difference (95% CI)[b] compared with the reference category		
	Difference in coagulation factor levels (95% CI)[a] per SD of HTGC (2.89%)[c]	HTGC Quartile 1 <1.34%[c] (29%)	HTGC Quartile 2 1.34%-2.66%[c] (26%)	HTGC Quartile 3 2.66%-6.27%[c] (23%)	HTGC Quartile 4 ≥6.27%[c] (22%)
Fibrinogen (mg/dL)					
Model 1	7.8 (3.8, 11.8)	281	10.5 (-1.3, 22.3)	19.4 (7.0, 31.8)	18.9 (7.6, 30.2)
Model 2	8.3 (4.2, 12.4)		10.0 (-1.7, 21.7)	20.8 (7.2, 34.5)	21.0 (8.9, 33.1)
Model 3	7.8 (3.5, 12.1)		11.5 (0, 23.0)	20.6 (7.4, 33.8)	22.5 (9.9, 35.1)
Model 4	-2.8 (-7.6, 2.1)		-2.2 (-12.6, 9.2)	0.8 (-13.5, 15.0)	-9.4 (-25.9, 7.0)
Factor VIII (IU/dL)					
Model 1	3.6 (1.2, 6.1)	119	5.3 (-2.2, 12.8)	5.3 (-1.9, 12.5)	8.9 (1.7, 16.0)
Model 2	3.4 (0.9, 5.8)		4.4 (-3.3, 12.0)	4.0 (-3.9, 11.9)	7.2 (0, 14.3)
Model 3	3.5 (1.0, 6.0)		4.7 (-3.2, 12.6)	4.9 (-3.0, 12.7)	8.4 (1.4, 15.4)
Model 4	1.8 (-1.2, 4.9)		1.8 (-6.8, 10.4)	0.5 (-8.1, 9.2)	3.5 (-6.3, 13.3)
Factor IX (IU/dL)					
Model 1	9.4 (7.9, 10.8)	102	11.3 (8.0, 14.7)	18.4 (14.5, 22.2)	25.7 (21.5, 29.8)
Model 2	9.5 (8.0, 10.9)		11.0 (7.8, 14.3)	19.1 (14.8, 23.5)	25.6 (21.6, 29.5)
Model 3	9.8 (8.2, 11.3)		11.7 (8.5, 15.0)	20.0 (16.0, 24.0)	27.1 (22.9, 31.2)
Model 4	6.2 (4.4, 8.1)		8.0 (4.8, 11.1)	12.5 (8.5, 16.6)	19.1 (12.6, 25.6)

Supplementary Table 4. (continued)

	Continuous scale	Reference (mean levels)	Mean difference (95% CI)[b] compared with the reference category		
	Difference in coagulation factor levels (95% CI)[a] per SD of HTGC (2.89%)[c]	**HTGC Quartile 1 <1.34%[c] (29%)**	**HTGC Quartile 2 1.34%-2.66%[c] (26%)**	**HTGC Quartile 3 2.66%-6.27%[c] (23%)**	**HTGC Quartile 4 ≥6.27%[c] (22%)**
Factor XI (IU/dL)					
Model 1	2.2 (0.8, 3.6)	111	6.3 (1.9, 10.6)	6.7 (2.3, 11.1)	7.0 (3.1. 10.9)
Model 2	3.1 (1.7, 4.5)		6.9 (2.6, 11.2)	8.8 (4.2, 13.4)	9.2 (5.3, 13.1)
Model 3	3.2 (1.7, 4.8)		7.2 (2.8, 11.5)	9.5 (4.5, 14.4)	10.1 (5.8, 14.3)
Model 4	1.4 (-0.4, 3.2)		5.4 (0.8, 9.9)	4.8 (-0.7, 10.2)	6.7 (0.9, 12.5)

Results were based on analyses weighted towards a normal body mass index distribution. Data were missed for some participants in some subgroups.

CI, confidence interval; HTGC, hepatic triglyceride content; SD, standard deviation.

[a] Beta coefficients (95% CI) from linear regression per weighted SD in natural log-transformed HTGC.

[b] Beta coefficients (95% CI) obtained by linear regression in each weighted quartile of natural log-transformed HTGC compared with the lowest quartile (reference category).

[c] Values of SD and quartiles of HTGC were back transformed for interpretation.

Model 1: crude association.

Model 2: adjustment for age and sex.

Model 3: model 2 + adjustment for ethnicity, education level, alcohol intake, physical activity, tobacco smoking, estrogen use and menopause status.

Model 4: model 3 + adjustment for visceral adipose tissue and total body fat.

Supplementary Table 5. Association between hepatic triglyceride content and coagulation factor levels in 1326 participants from the NEO study with alcohol consumption <20g/day according to sex

	Reference (mean levels)	Mean difference (95% CI)[a] compared with the reference category		
	HTGC Quartile 1 <1.34%[b]	**HTGC Quartile 2 1.34%-2.66%[b]**	**HTGC Quartile 3 2.66%-6.27%[b]**	**HTGC Quartile 4 ≥6.27%[b]**
Fibrinogen (mg/dL)				
Model 1				
Men	265	20.8 (-3.0, 44.7)	26.7 (4.5, 48.9)	23.4 (2.9, 44.0)
Women	287	9.0 (-4.3, 22.3)	23.4 (7.7, 39.2)	26.2 (11.2, 41.3)
Model 2				
Men	265	22.2 (-1.2, 45.5)	23.0 (0.3, 45.7)	21.6 (1.0, 42.2)
Women	287	5.7 (-7.9, 19.2)	19.8 (2.6, 37.1)	20.1 (5.4, 34.9)
Model 3				
Men	265	27.5 (5.2, 49.9)	26.5 (4.2, 48.9)	24.1 (4.2, 44.0)
Women	287	5.2 (-8.1, 18.5)	18.4 (1.3, 35.4)	23.2 (8.4, 38.1)
Model 4				
Men	265	13.6 (-8.1, 35.2)	11.0 (-12.9, 34.9)	2.3 (-23.1, 27.6)
Women	287	-9.1 (-22.5, 4.4)	-6.7 (-24.5, 11.0)	-13.7 (-33.4, 6.0)
Factor VIII (IU/dL)				
Model 1				
Men	119	2.5 (-9.2, 14.2)	-1.8 (-8.8, 12.4)	6.7 (-4.7, 18.0)
Women	119	6.7 (-2.8, 16.3)	8.6 (-1.4, 18.5)	10.8 (1.9, 19.7)
Model 2				
Men	119	2.9 (-8.8, 14.5)	0.4 (-10.2, 11.1)	5.4 (-5.6, 16.5)
Women	119	4.9 (-5.2, 14.9)	6.0 (-5.2, 17.1)	8.3 (-1.4, 18.0)
Model 3				
Men	119	3.4 (-7.5, 14.4)	0 (-9.6, 9.5)	5.8 (-3.9, 15.2)
Women	119	4.2 (-6.0, 14.5)	6.2 (-4.4, 16.7)	9.9 (1.1, 18.8)
Model 4				
Men	119	3.0 (-8.4, 14.3)	0.2 (-11.2, 11.5)	6.3 (-8.9, 21.5)
Women	119	0.7 (-10.8, 12.2)	-1.8 (-13.1, 9.5)	1.7 (-10.4, 13.7)
Factor IX (IU/dL)				
Model 1				
Men	101	13.5 (7.8, 19.1)	15.2 (9.8, 20.6)	24.7 (17.4, 31.9)
Women	102	10.3 (6.2, 14.4)	22.0 (16.3, 27.8)	27.3 (22.7, 31.9)
Model 2				
Men	101	13.7 (8.1, 19.4)	14.2 (9.1, 19.4)	23.9 (16.9, 31.0)
Women	102	10.4 (6.2, 14.5)	22.1 (15.9, 28.4)	26.8 (22.1, 31.4)
Model 3				
Men	101	14.0 (8.8, 19.3)	13.5 (8.5, 18.6)	23.2 (15.3, 31.0)
Women	102	10.5 (6.4, 14.6)	23.0 (17.7, 28.3)	29.6 (25.4, 33.8)
Model 4				
Men	101	11.9 (6.9, 16.8)	8.6 (2.8, 14.3)	18.9 (6.1, 31.7)
Women	102	5.9 (1.9, 9.8)	13.4 (8.4, 18.3)	19.1 (12.7, 25.5)

Supplementary Table 5. (continued)

	Reference (mean levels)	Mean difference (95% CI)[a] compared with the reference category		
	HTGC Quartile 1 <1.34%[b]	**HTGC Quartile 2 1.34%-2.66%[b]**	**HTGC Quartile 3 2.66%-6.27%[b]**	**HTGC Quartile 4 ≥6.27%[b]**
Factor XI (IU/dL)				
Model 1				
Men	106	6.5 (-0.9, 13.9)	5.2 (-0.9, 11.4)	6.8 (0.9, 12.6)
Women	113	7.6 (2.5, 12.8)	11.9 (5.8, 17.9)	11.1 (6.0, 16.3)
Model 2				
Men	106	6.4 (-1.0, 13.7)	5.3 (-1.0, 11.6)	7.3 (1.5, 13.1)
Women	113	6.4 (1.1, 11.6)	10.6 (4.2, 17.0)	9.7 (4.4, 15.0)
Model 3				
Men	106	7.5 (0.1, 14.9)	4.4 (-2.3, 11.1)	6.6 (0.6, 12.7)
Women	113	6.6 (1.5, 11.8)	12.2 (5.8, 18.7)	12.8 (7.4, 18.2)
Model 4				
Men	106	7.3 (-0.6, 15.2)	0.2 (-7.5, 7.8)	2.7 (-4.2, 9.6)
Women	113	3.9 (-1.4, 9.2)	7.1 (0, 14.2)	11.0 (3.3, 18.7)

Results were based on weighted analyses towards a normal body mass index distribution. Data were missed for some participants in some subgroups. HTGC, hepatic triglyceride content; CI, confidence interval.

[a] Beta coefficients (95% CI) obtained by linear regression in each weighted quartile of natural log-transformed HTGC compared with the lowest quartile (reference category).

[b] Values of HTCG quartiles were back transformed for interpretation.

Model 1: crude association. Model 2: adjustment for age. Model 3: model 2 + adjustment for ethnicity, education level, alcohol intake, physical activity, tobacco smoking, estrogen use (women only) and menopause status (women only). Model 4: model 3 + adjustment for visceral adipose tissue and total body fat.

Chapter 7

Summary and General discussion

The aim of this thesis was to investigate the associations of traditional cardiometabolic risk factors with risk of a first and recurrent venous thrombosis. Additionally, associations between lipids and levels of several hemostatic factors were assessed. This chapter provides an overview of the main findings, and addresses some methodological considerations. Furthermore, the clinical implications of the thesis' findings and directions for future research will be discussed.

OVERVIEW OF MAIN FINDINGS

Lipid levels and risk of a first venous thrombosis

In **chapter 2**, the association between lipid levels and risk of a first venous thrombosis was assessed in the MEGA study. In contrast to arterial cardiovascular disease (CVD), levels of major lipids, i.e. total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides, were not associated with risk of venous thrombosis. However, decreasing levels of apolipoprotein (apo) B and apo A1 were associated with an increased risk of venous thrombosis in a dose-response fashion, also after adjustment for potential confounders. Although apo B and to a lesser extent apo A1 levels were related to levels of several hemostatic factors and C-reactive protein, none of these factors explained the association of apolipoproteins B and A1 with risk of venous thrombosis.

While there is strong evidence that elevated apo B and LDL-C levels are associated with an increased risk for arterial CVD [1,2], we observed an inverse relationship between apo B levels and risk of venous thrombosis. Since venous and arterial CVD have different pathophysiology (see Fig.1 chapter 1), we hypothesized that apo B may play different roles in the mechanisms that are at the basis of venous thrombosis versus arterial CVD. Indeed, our findings on the protective role of apo B and apo A1 against venous thrombosis are in line with experimental data, in which both apolipoproteins have been suggested to have anticoagulant properties [3-6].

On the basis of our results, dyslipidemia does not explain the relationship between venous thrombosis and increased risk of subsequent arterial CVD, as none of the major lipids was associated with risk of venous thrombosis. In addition, risk estimates for venous thrombosis associated with apo B levels pointed in the opposite direction to that reported in arterial disease. One might argue that our findings on apo B and venous thrombosis are not consistent with the putative protection conferred by statins against venous thrombosis, since these drugs decrease apo B levels [7,8]. It is noteworthy that statins may have antithrombotic effects that are unrelated to their lipid-lowering activity, such as downregulation of tissue factor [9]. It is currently unknown, however, to what extent the antithrombotic potential of statins would influence the thrombosis risk or the anticoagulant properties of apo B demonstrated *in vitro*.

Since the study design used to answer our research question was a case-control study, we cannot exclude the possibility of reverse causation, i.e. that the venous thrombotic event has led to changes in lipid levels. Indeed, as discussed in **chapter 2**, patients may have modified certain lifestyle factors after the event of venous thrombosis, which could have affected their lipid profile. Randomized clinical trials published during the nineties showed that changes in diet affected lipid levels in individuals with dyslipidemia. However, the reduction in TC, LDL-C and apo B levels produced by a low-fat diet taken from 9 weeks to 1 year in these trials was small or virtually absent without concomitant statin use or aerobic-exercise program. It is quite unlikely that venous thrombosis patients would systematically adhere to a low-fat diet in addition to statin use and/or exercise after the thrombotic event, especially considering that such recommendation is not routinely given, in contrast to arterial disease. Another mechanism for reverse causation would be the effect of acute phase reactions brought about by the thrombotic event on lipid levels. During acute phase reactions in humans, triglyceride levels typically increase and HDL-C and apo A1 levels decrease, whereas TC, LDL-C and apo B levels can either decrease or do not change. Nevertheless, this is an unlikely mechanism for reverse causation, since blood was collected with a median of 10 months after the thrombotic event, by which time the effects of the acute-phase reaction will have worn off. Finally, changes in lipid levels in our study population owing to reverse causation would be expected to affect not only the direction of the point estimates related to the apolipoproteins but also to the other lipids, such as TC, which also makes it unlikely that our findings can be due to this explanation.

Clinical implications and directions for future research

It is not possible to suggest directly new approaches for preventing or treating venous thrombosis based on our findings, apart from exclusion of most lipids from risk factor profiles. Still, our results on the inverse association between apolipoprotein B and A1 levels and risk of venous thrombosis may form the basis for further studies to confirm our findings, as well as to assess the physiological relevance of the anticoagulant properties of apo B and apo A1, and to determine the mechanism, whether or not causal, underlying the link between these apolipoproteins and venous thrombosis. A deep understanding on the possible mechanisms by which apolipoproteins B and A1 could affect venous thrombosis risk might provide insight to the development of novel strategies to prevent venous thrombosis at a minimum cost of bleeding risk.

Cardiometabolic risk factors and risk of recurrent venous thrombosis

The role of cardiometabolic risk factors in assessing risk of recurrent venous thrombosis has not been extensively studied.

In **chapter 3**, we evaluated the associations between levels of several lipids (i.e. TC, triglycerides, LDL-C, HDL-C, apo A1 and apo B) and risk of recurrent venous thrombosis. None of the lipids studied identified patients at an increased risk of recurrence, including those with unprovoked first events. In **chapter 4**, we further assessed the association of estimated glomerular filtration rate (eGFR), glucose levels and hematologic variables (i.e. blood cell count) with recurrence. We found that testing for glucose levels and hematologic variables did not identify patients at increased risk of recurrent venous thrombosis, including those with unprovoked first events, and an association between renal dysfunction, as measured by a decreased eGFR, and recurrence appeared to be slight at most.

It is important to address that in the MEGA study, levels of apolipoproteins B and A1 (**chapter 2** of this thesis), eGFR [10], red cell distribution width [11], and monocyte count [11] were all associated with a first venous thrombosis. However, in the MEGA follow-up study, a decreased eGFR was only marginally associated with recurrence, and no associations with recurrent events were observed for the other aforementioned factors. These results are consistent with the fact that risk factors for a first event do not necessarily predict recurrence [12,13]. For instance, inherited thrombophilia, a classic risk factor for a first event, only poorly predicts recurrence in unselected patients [12].

Clinical implications and directions for future research

Testing for lipid levels, glucose levels, and hematologic variables did not identify patients at increased risk of recurrent venous thrombosis, and these should not influence decisions on duration of anticoagulant treatment. Renal dysfunction appeared to predict recurrence to some extent. Still, given the well-established increased risk of major bleeding in patients with renal dysfunction during anticoagulant treatment [14,15], studies with larger samples sizes, using a uniform measurement of kidney function, are needed to allow a detailed assessment of the ability of a decreased eGFR to identify patients at increased risk of recurrent venous thrombosis, in particular, those with unprovoked first events.

Association between lipid and hemostatic factor levels

The interrelation between hemostatic factor and lipid levels is not known in detail, as studies on this topic are few, involve small sample sizes, and study a limited number of lipids and hemostatic factors. In **chapter 5**, we investigated how levels of several hemostatic factors (procoagulant, anticoagulant, and fibrinolytic factors) were interrelated, forming clusters, and how these clusters related to lipid levels in 2874 population controls. Among our main findings is that vitamin K-dependent factors (VKDFs), including procoagulant (factor II, factor VII, factor IX, and factor X)

and anticoagulant (protein C and protein S) factors, clustered together. Upon addition of lipids to the analysis, all VKDFs consistently clustered with triglyceride levels.

In **chapter 6**, we studied the relationship between liver fat content, measured as hepatic triglyceride content (HTGC), and levels of coagulation factors associated with an increased risk of venous thrombosis. We found that fibrinogen, factor VIII, factor IX, and factor XI levels increased dose-dependently across HTGC quartiles, even after adjustment for several demographic and lifestyle potential confounding factors. However, with further adjustment for total body and visceral fat, the associations between HTGC and levels of fibrinogen, factor VIII and factor XI disappeared, whereas the associations between HTGC and factor IX levels, albeit attenuated, persisted, as did the dose-response relation. This observation could be relevant, as high levels of factor IX related to HTGC have the potential to be a critical pathway by which obesity increases the risk of venous thrombosis.

Clinical implications and directions for future research

Taken together, results from both studies support the existence of a close association between VKDFs, including factor IX, and triglycerides. One may speculate that common mechanisms lying outside the genes coding for VKDFs and triglycerides could explain, at least in part, this association. Clarification of this issue is worth pursuing, as therapeutic strategies targeting possible common regulatory mechanisms of VKDFs and lipids, in particular, triglycerides, might have the potential to decrease the risk of both venous thrombosis and arterial CVD.

CONCLUSIONS

This thesis provided epidemiological evidence that some traditional cardiometabolic risk factors are related to first and recurrent venous thrombosis. Most lipids were not associated with risk of a first or recurrent venous thrombosis. In contrast, decreasing levels of apolipoproteins B and apo A1 were associated with an increased risk of first venous thrombosis, which we could not explain through several proposed mechanisms, such as confounding or other non-causal explanations, or through mediation via inflammation or changes in the hemostatic factors. Overall, testing for cardiometabolic risk factors does not seem useful to identify patients at an increased risk of recurrence. In addition, vitamin K-dependent factor levels are associated with both serum and hepatic triglycerides. Future research should focus on clarifying the possible mechanisms underlying this association.

REFERENCES

1. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. JAMA 2009; 302: 1993-2000.
2. Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. Lancet 2012; 380: 572-580.
3. Ettelaie C, Howell RM. The inhibition of thromboplastin by apolipoprotein-B and the effect of various associated lipids. Thromb Res 1992; 68: 175-184.
4. Ettelaie C, Wilbourn BR, Adam JM, James NJ, Bruckdorfer KR. Comparison of the inhibitory effects of ApoB100 and tissue factor pathway inhibitor on tissue factor and the influence of lipoprotein oxidation. Arterioscler Thromb Vasc Biol 1999; 19: 1784-1790.
5. Brill A, Yesilaltay A, De Meyer SF, Kisucka J, Fuchs TA, Kocher O, Krieger M, Wagner DD. Extrahepatic high-density lipoprotein receptor SR-BI and apoA-I protect against deep vein thrombosis in mice. Arterioscler Thromb Vasc Biol 2012; 32: 1841-1848.
6. Fernandez JA, Deguchi H, Banka CL, Witztum JL, Griffin JH. Re-evaluation of the anticoagulant properties of high-density lipoprotein-brief report. Arterioscler Thromb Vasc Biol 2015; 35: 570-572.
7. Colhoun HM, Betteridge DJ, Durrington PN, Hitman GA, Neil HA, Livingstone SJ, Thomason MJ, Mackness MI, Charlton-Menys V, Fuller JH; CARDS investigators. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. Lancet 2004; 364: 685-96.
8. Mora S, Caulfield MP, Wohlgemuth J, Chen Z, Superko HR, Rowland CM, Glynn RJ, Ridker PM, Krauss RM. Atherogenic Lipoprotein Subfractions Determined by Ion Mobility and First Cardiovascular Events After Random Allocation to High-Intensity Statin or Placebo: The Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) Trial. Circulation 2015; 132: 2220-9.
9. Violi F, Calvieri C, Ferro D, Pignatelli P. Statins as antithrombotic drugs. Circulation. 2013; 127: 251-257.
10. Ocak G, Vossen CY, Lijfering WM, Verduijn M, Dekker FW, Rosendaal FR, Cannegieter SC. Role of hemostatic factors on the risk of venous thrombosis in people with impaired kidney function. Circulation 2014;129: 683-91.
11. Rezende SM, Lijfering WM, Rosendaal FR, Cannegieter SC. Hematologic variables and venous thrombosis: red cell distribution width and blood monocyte count are associated with an increased risk. Haematologica 2014; 99: 194-200.
12. Cannegieter SC, van Hylckama Vlieg A. Venous thrombosis: understanding the paradoxes of recurrence. J Thromb Haemost 2013; 11 Suppl 1:161-9.
13. Dahabreh IJ, Kent DM. Index event bias as an explanation for the paradoxes of recurrence risk research. JAMA 2011; 305: 822-3.

14. Kearon C, Akl EA, Ornelas J, Blaivas A, Jimenez D, Bounameaux H, Huisman M, King CS, Morris TA, Sood N, Stevens SM, Vintch JR, Wells P, Woller SC, Moores L. Antithrombotic Therapy for VTE Disease: CHEST Guideline and Expert Panel Report. Chest 2016; 149: 315-52.

15. Kooiman J, den Exter PL, Cannegieter SC, le Cessie S, del Toro J, Sahuquillo JC, Pedrajas JM, Huisman MV. Impact of chronic kidney disease on the risk of clinical outcomes in patients with cancer-associated venous thromboembolism during anticoagulant treatment. J Thromb Haemost 2013; 11: 1968-76.

Chapter 8

Nederlandse samenvatting

Acknowledgements

Curriculum Vitae

List of publications

NEDERLANDSE SAMENVATTING

Veneuze trombose (waaronder diepe veneuze trombose en longembolie wordt verstaan) is een aandoening die optreedt bij ongeveer 1 tot 2 per 1000 personen per jaar. Veneuze trombose is de derde meest voorkomende cardiovasculaire ziekte ter wereld, en heeft een grote impact op de gezondheidszorg aangezien de ziekte vaak leidt tot hospitalisatie, restschade en mortaliteit. Tot de lange termijn complicaties van veneuze trombose behoren het post-trombotisch syndroom (pijnlijk en zwaar aanvoelend, ulcererend been) en chronische pulmonale hypertensie (kortademigheidsklachten). Veneuze trombose is een multicausale ziekte waarmee bedoeld wordt dat een opeenstapeling van genetische en omgevingsfactoren uiteindelijk leidt tot trombosevorming. Ondanks dat vele risicofactoren bekend zijn, blijft de reden van optreden van veneuze trombose in 50% van de gevallen onverklaard. Om deze reden wordt voortdurend gezocht naar nieuwe risicofactoren van veneuze trombose. Mochten deze risicofactoren worden gevonden, kan men vervolgens interveniëren op die risicofactor om daarmee de kans tot het optreden van veneuze trombose te doen verminderen. Verscheidene studies hebben in het verleden laten zien dat mensen met veneuze trombose een hoger risico hebben op het krijgen van arteriële cardiovasculaire aandoeningen (zoals hartinfarct of beroerte). De reden waarom deze twee ziektebeelden met elkaar gerelateerd zijn is onduidelijk, maar gesuggereerd wordt dat het te maken heeft met onderliggende risicofactoren die zowel een hoger risico voor veneuze trombose - als voor arteriële cardiovasculaire aandoeningen geven. Zo is overgewicht, een traditionele cardiometabole risicofactor, geassocieerd met zowel veneuze trombose als met arteriële cardiovasculaire aandoeningen. Echter, de relatie tussen andere cardiometabole risicofactoren en veneuze trombose is minder duidelijk. Ook is het onduidelijk of cardiometabole risicofactoren gerelateerd zijn met componenten van de stollingscascade. Inzicht hierin zou onze kennis kunnen verbreden over *hoe* deze cardiometabole risicofactoren veneuze trombose kunnen doen laten ontstaan. Het hoofddoel van dit proefschrift is om te bestuderen of er een associatie is tussen traditionele cardiometabole risicofactoren, zoals dyslipidemie, diabetes, en gestoorde nierfunctie en het optreden van (recidief) veneuze trombose. Een onderliggend doel is om voor verscheidene lipiden-profielen het mogelijk onderliggende pathofysiologische mechanisme tot op stollingsniveau te verklaren.

In **hoofdstuk 2** werd de associatie tussen dyslipidemie en het risico op een eerste veneuze trombose onderzocht in de 'Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis' (MEGA) studie. Het mogelijke onderliggende mechanisme werd tot op stollingsniveau bekeken, waarbij eerst werd gecorrigeerd voor mogelijk onderliggende verklarende factoren (confounding), en vervolgens voor stollingsfactoren en inflammatie. Resultaten uit deze studie laten zien dat

totaal cholesterol (TC), 'low-density lipoproteine' cholesterol (LDL-C), 'high-density lipoproteine' cholesterol (HDL-C) en triglyceriden niet geassocieerd zijn met een verhoogd risico op veneuze trombose. Echter verlaagde waarden van apolipoproteine B en A1 zijn wel dosis-respons geassocieerd met een hoger risico op veneuze trombose, welke associatie blijft bestaan na correctie voor confounding. Ofschoon apolipoproteine B en A1 gerelateerd waren met verschillende stollingsfactoren en inflammatie (i.e. C-reactief proteïne), kon geen van deze factoren de associatie tussen apolipoproteinen en veneuze trombose verklaren. Op basis van deze bevindingen is het onwaarschijnlijk dat dyslipidemie de relatie tussen veneuze trombose en arteriële cardiovasculaire aandoeningen doet verklaren aangezien geen van de traditionele lipiden (TC, LDL-C, HDL, C, triglyceriden) met veneuze trombose waren geassocieerd. Daarbij was de relatie tussen apolipoproteine B en A1 en veneuze trombose een inverse relatie (i.e. precies omgekeerd ten opzichte van de relatie tussen apolipoproteine B en A1 en arteriële cardiovasculaire aandoeningen). Interessant is echter dat de inverse relatie tussen apolipoproteine B en A1 en veneuze trombose consistent is met bevindingen uit experimentele studies waarbij hoge waarden van apolipoproteine B en A1 leiden tot verhoogde antistollingseffecten.

In **hoofdstuk 3** werd de rol van de lipiden TC, triglyceriden. LDL-C, HDL-C, en apolipoproteine B en A1 in relatie tot het optreden van een recidief veneuze trombose onderzocht in de MEGA follow-up studie. In **hoofdstuk 4** werd een soortgelijke studie uitgevoerd, waarbij nu de vraag was of gestoorde nierfunctie (gemeten met 'estimated glomerular filtration rate' [eGFR), glucose spiegels en hematologische bloedparameters (zoals hemoglobine) geassocieerd zijn met recidief veneuze trombose. De conclusies uit beide hoofdstukken zijn dat deze bloedparameters niet geassocieerd zijn met een hogere kans op het krijgen van een recidief veneuze trombose. Dit geldt zowel voor patiënten met een eerste uitgelokte veneuze trombose, als voor patiënten met een eerste onverklaarde veneuze trombose. Deze bloedparameters zijn daarom niet bruikbaar als gegevens voor de arts om te bepalen wie wellicht gebaat zou zijn met een langdurige behandeling met antistollingsmiddelen om daarmee het hoge risico op recidief veneuze trombose te doen verlagen. Eén uitzondering is wellicht eGFR waarbij gezien werd dat patiënten met lage waarden van eGFR een iets hoger risico hadden op het krijgen van een recidief veneuze trombose. Echter, omdat deze patiënten ook een hoog risico hebben op het krijgen van bloedingen onder antistollingsgebruik, is het onduidelijk of deze patiënten al dan niet baat hebben van langdurige behandeling met antistollingsmiddelen.

In **hoofdstuk 5** werd de relatie tussen stollingsfactoren (procoagulante-, anticoagulante- en fibrinolytische stollingsfactoren) en lipidewaarden onderzocht in 2874 gezonde controle personen uit de MEGA studie. De belangrijkste bevinding uit deze studie is dat de vitamine K afhankelijke stollingsfactoren (II, VII, IX, X en proteïne

C en proteïne S) groeperen met triglyceriden. In **hoofdstuk 6** werd de associatie tussen levervetwaarden, gemeten als 'hepatic triglyceride content' (HTGC), en verschillende stollingsfactoren onderzocht in de Netherlands Epidemiology of Obesity (NEO) studie. Gevonden werd dat alle gemeten stollingsfactoren (fibrinogeen, factor VIII, factor IX en factor XI) hoger waren in mensen in wie de levervetwaarden het hoogst waren. Deze relatie bleef aanwezig nadat de bevindingen gecorrigeerd waren voor verschillende demografische variabelen en levensstijl. Echter na correctie voor totaal lichaamsvet en visceraal vet verdween de relatie voor de stollingsfactoren fibrinogeen, factor VIII en factor XI. Voor factor IX bleef echter ook na deze correctie de associatie met levervet bestaan. Deze bevinding is potentieel relevant omdat factor IX een verklaring zou kunnen geven waarom mensen met obesitas en verhoogde HGTC waarden een hoger risico hebben op het krijgen van veneuze trombose. Verder ondersteunen de resultaten uit hoofdstuk 5 en 6 de hypothese dat de vitamine K afhankelijke stollingsfactoren, waaronder factor IX, en triglyceriden sterk met elkaar geassocieerd zijn. Therapie tegen hoge triglyceriden waarden (bv statine) zou hierom mogelijk ook een antistollende werking kunnen hebben (verlaging factor IX).

Ter conclusie, dit proefschrift heeft met behulp van epidemiologische studies aangetoond dat sommige cardiometabole risicofactoren geassocieerd zijn met eerste veneuze trombose. Voor recidief veneuze trombose konden weinig tot geen aanwijsbare metabole risicofactoren gevonden worden die het risico op het optreden van een nieuwe trombose konden voorspellen. Deze laatste bevinding maakt het onwaarschijnlijk dat testen op cardiometabole risicofactoren kan leiden tot een betere risicostratificatie van patiënten met veneuze trombose en het optreden van een recidief veneuze trombose. Daarnaast heeft dit proefschrift aangetoond dat vitamine K afhankelijke stollingsfactoren geassocieerd zijn met triglyceriden en levervet. Toekomstige studies dienen uit te wijzen wat het precieze mechanisme is die deze bevinding verklaart en hoe deze te duiden in het kader van farmacotherapie.

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CURRICULUM VITAE

Vânia Maris Morelli was born on March 1, 1969, in São Paulo, Brazil. She started in the Brazilian private education system in 1975. In 1986, she graduated from high school at Colégio Civitatis in São Paulo. In 1987, Vânia commenced her medical studies at Faculdade de Medicina da Fundação do ABC, and graduated in 1992. From 1993 to 1996, she attended the Medical Residency Programme in Internal Medicine and in Hematology at Universidade Federal de São Paulo. Vânia's interest in the field of epidemiology of venous thrombosis started during her PhD in Brazil at Universidade Federal de São Paulo. The PhD was finished in 2000, under the supervision of Dr. D.M. Lourenço, with the thesis entitled "Evaluation of inherited thrombophilia and hyperhomocysteinemia in patients with venous thrombosis". In order to have a deeper contact with research on venous thrombosis, Vânia performed an internship from 2002 to 2003 (sponsored by FAPESP, Brazil) at the Department of Thrombosis and Hemostasis of the Leiden University Medical Center (LUMC), the Netherlands, under the supervision of Prof. dr. R.M. Bertina. During the profitable period of this internship, Vânia also had the opportunity to meet Prof. dr. F.R. Rosendaal from the Department of Clinical Epidemiology of the LUMC, and the privilege to discuss with him the results of the research project. After returning to Brazil, even though Vânia had to dedicate most of her career working as a clinical hematologist, her interest in research on venous thrombosis persisted over the years. Because of her growing interest in studying the epidemiology of venous thrombosis, she decided to do a career change in 2014, and be focused on research only. To accomplish this goal, she started a second internship at the LUMC in 2014, now at the Department of Clinical Epidemiology, which became the starting point for this thesis. During the internship, she had the opportunity to start on the PhD Programme of the Department of Clinical Epidemiology (2014) under the supervision of Prof. dr. S.C. Cannegieter, Prof. dr. F.R. Rosendaal, and Dr. W.M. Lijfering. She spent the following years studying topics related to cardiometabolic risk factors, blood coagulation and risk of venous thrombosis, which are now described in this thesis. In addition, she took courses in Clinical Epidemiology, and presented her work at international conferences. Since July 2017, she has been working as a researcher at the K.G. Jebsen Thrombosis Research and Expertise Center (TREC), University of Tromsø, Norway. Vânia's main career goal is to continue being focused on research in the field of epidemiology of venous thrombosis.

LIST OF PUBLICATIONS

Morelli VM, De Visser MC, Vos HL, Bertina RM, Rosendaal FR. ABO blood group genotypes and the risk of venous thrombosis: effect of factor V Leiden. J Thromb Haemost 2005; 3:183-5.

Morelli VM, de Visser MC, van Tilburg NH, Vos HL, Eikenboom JC, Rosendaal FR, Bertina RM. ABO blood group genotypes, plasma von Willebrand factor levels and loading of von Willebrand factor with A and B antigens. Thromb Haemost 2007; 97: 534-41.

Morelli VM, Lijfering WM, Rosendaal FR, Cannegieter SC. Lipid levels and risk of recurrent venous thrombosis: results from the MEGA follow-up study. J Thromb Haemost 2017; 15: 695-701.

Morelli VM, Lijfering WM, Bos MHA, Rosendaal FR, Cannegieter SC. Lipid levels and risk of venous thrombosis: results from the MEGA-study. Eur J Epidemiol 2017; 32: 669-81.

Morelli VM, Lijfering WM, Rezende SM, van Hylckama Vlieg A, Rosendaal FR, Cannegieter SC. Role of routine laboratory tests in assessing risk of recurrent venous thrombosis: results from the MEGA follow-up study. *To be submitted*

Morelli VM, Li-Gao R, Lijfering WM, Rosendaal FR, Cannegieter SC, van Hylckama Vlieg A. Interrelation between levels of hemostatic factors, lipids and c-reactive protein in population controls. *To be submitted*

Morelli VM, de Mutsert R, de Roos A, Lamb HJ, van Hylckama Vlieg A, Bos MHA, Rosendaal FR, Lijfering WM, Cannegieter SC. Association between hepatic triglyceride content and coagulation factor levels: The Netherlands Epidemiology of Obesity Study. *To be submitted*

Li-Gao R, **Morelli VM**, Lijfering WM, Cannegieter SC, Rosendaal FR, van Hylckama Vlieg A. The role of fasting glucose levels and self-reported diabetes in the risk of a first event of venous thrombosis: the MEGA case-control study. *To be submitted*