Blood Donation *And* Cardiovascular Disease

Addressing the Healthy Donor Effect

Karlijn Peffer

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Promotoren
Prof. dr. A.L.M. Verbeek
Prof. dr. M. den Heijer (VUmc)

Copromotor
Dr. F. Atsma

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Chapter 1

Motive and Outline

The incidence of cardiovascular disease is increasing in the western world, contributing up to 20.5% of the total burden of disease in the Netherlands in 2011, and with 38,371 fatalities being the second cause of death in 2012. Meanwhile, blood donation is a common practice in the Netherlands. In 2012, 293,839 people donated whole-blood or plasma. This is approximately 2.7% of the Dutch population that is within the age-appropriate range (18 - 65 years) for blood donation. Although being quite common, not much is known about the physiological effects of blood donation besides its effect on iron stores [1–3]. Lower iron stores have been hypothesized to decrease cardiovascular disease. Since blood donation is able to decrease iron stores tremendously, the research set out in this thesis addresses whether blood donation is able to reduce cardiovascular disease.

1.1 Cardiovascular Disease and Iron

A stabilising cardiovascular epidemic

Until 2004 in men and 2011 in women, cardiovascular disease was the leading cause of death in the Netherlands (Figure 1.1). It has been competing with cancer, but both diseases now seem to have reached a new equilibrium with approximately 40,000 deaths each year. The decreasing trend of cardiovascular death has mainly been attributed to the increased awareness, detection, and improved preventive actions such as lifestyle changes, but most of all to improved therapies. Unfortunately, the success in health care has taken its toll on the incidence of cancer now that patients can survive the initial cardiovascular event and it has become a chronic disease.

The entire spectrum of cardiovascular diseases (CVD) covers a wide range from more chronic conditions such as atherosclerosis that can ultimately lead to more acute phenomena such as acute myocardial infarction or stroke. Atherosclerosis is a continuing inflammatory process of endothelial accumulation of white blood cells that results in the attraction and infiltration of monocytes that turn into macrophages once inside the intima [4–6]. There, they scavenge oxidized LDL-cholesterol and turn into foam cells, forming fatty streaks. The subsequent migration of smooth cells from the intima to the endothelium under the influence of cytokines and growth factors results in the formation of a fibrous cap. Further calcium deposits and accumulated debris finalized the plaque formation, which reduces the blood flow in the artery. When the fibrous cap of a vulnerable plaque ruptures, thrombogenic material such as collagen can leave the plaque, enhancing thrombus formation in the arterial lumen, and eventually sends off a thromboembolism.

Classic risk factors of CVD include hypertension, dyslipidaemia, smoking, diabetes, and obesity. Atherosclerosis can persist for several decades before becoming clinically manifest; the average age at hospital admissions of acute myocardial infarction is 65 years in men and 71 years in women, and for stroke the respective ages are 69 years and 72 years [7]. Of interest are the gender differences: per 100,000 inhabitants, the incidence of cardiovascular disease is higher in men for all age categories. Likewise, the average age at CVD-death is lower in men (77 years) than women (84 years).

The hypothesis

A causal role of iron in cardiovascular disease has been first proposed by Sullivan in the 1980s [8]. He arrived upon his "iron-heart hypothesis" as an explanation for several observations: (1) premenopausal women have the lowest cardiovascular disease risk, but after menopause and menstrual blood loss ceases, they rapidly increase in cardiovascular disease to approach that observed in men; (2) western civilizations have an increased cardiovascular disease risk compared to developing countries, where iron deficiency occurs more often due to malnutrition; (3) myocardial failure in iron storage disease such as hemochromatosis.

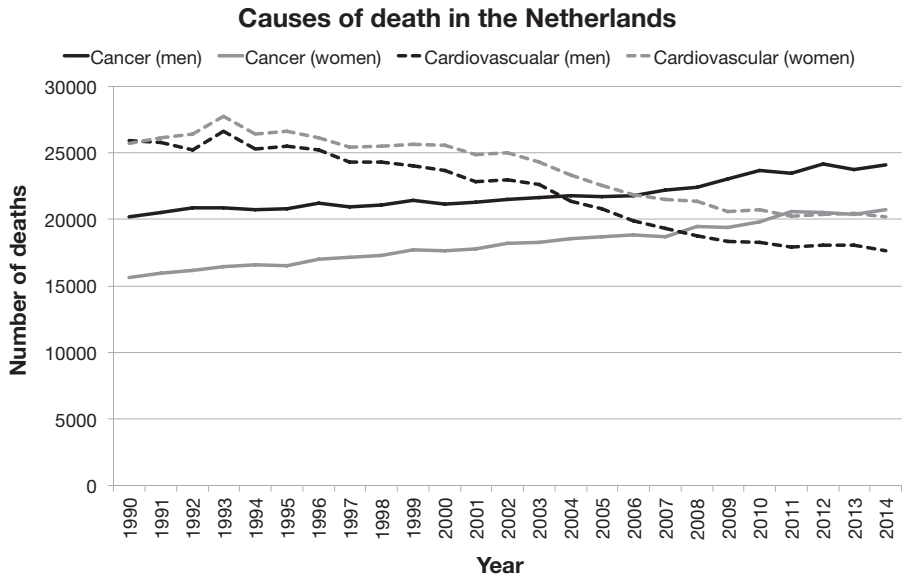


Figure 1.1: Sex-specific trends in the top-2 causes of death in the Netherlands (1990-2014)

Although the observation of myocardial failure in iron storage disease has become less established in the last few years, as patients with hereditary hemochromatosis, a condition accompanied by high levels of body iron stores, do not have an increased risk of atherosclerosis [9], the other two observations are still present today and are not yet fully explained by other phenomena. Indeed, the sex difference has been attempted to be explained by changing hormone levels during menopause and subsequent changes in lipid profile and body fat distribution [10–13], but since the failure of a large hormone replacement therapy trial (the Women's Health Initiative) in preventing cardiovascular disease [14], the iron hypothesis has gained importance.

Lowering iron stores

Iron is an important metal required for several metabolic processes in cells. Iron is found in two different cationic (or oxidative) states throughout the body: the more stabile ferric iron (Fe^{3+}) and the reactive ferrous iron (Fe^{2+}). When in ferrous state, better known as iron(II) oxide, it is at its most dangerous appearance and catalyzes the formation of reactive oxygen species (ROSs). Under aerobic conditions, Fe^{2+} reacts with oxygen resulting in Fe^{3+} and hydroxyl radicals ($OH^{\bullet -}$) according to the Fenton reaction: $Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + OH^{\bullet -}$. In turn, ferric iron can react with superoxide in the Haber-Weiss reaction

to produce yet again ferrous iron: Fe(III) + $O_2^{\bullet -} \longrightarrow$ Fe(II) + O_2. The resulting ROSs, most importantly the hydroxyl radical, can in turn oxidize and damage DNA and polyunsaturated fatty acids in lipids [15, 16]. ROSs play a role in the atherosclerotic process by oxidizing LDL-cholesterol and decrease the efflux of cholesterol in macrophages, thereby enhancing foam cell formation [17–19]. ROSs are also important contributors to impaired vascular reactivity through decreased NO synthesis and action, as well as their stimulating role in platelet aggregation and smooth muscle cell proliferation [20]. Furthermore, ROSs hamper insulin uptake and its biological intracellular activities, thereby disturbing glucose metabolism and vascular function [21].

Because of the catalytic properties of iron in the formation of reactive oxygen species, its level needs to be tightly controlled. In humans, iron is not actively secreted. Instead, total body iron content is regulated by the amount of dietary iron absorbed by the duodenal enterocyte and the subsequent release into the bloodstream by the only known cellular iron exporter ferroportin [22]. Uptake by the apical membrane requires the divalent metal transporter-1 (DMT-1) for dietery Fe^{3+} which is first reduced to Fe^{2+} by DcytB, and an unknown haeme transporter for dietary haeme iron [22]. The uptake rate of Fe^{3+} by DMT-1 can be enhanced by ascorbate (vitamin C) as this increases the reductase activity of DcytB.

A human body contains approximately 3000 to 4000 mg of iron. Most of this, around 2500 mg, is found in erythrocytes, where it is part of haemoglobin that facilitates the transportation of oxygen from the lungs to all other tissues. When erythrocytes become senescent after approximately 120 days, they are phagocytosed by reticuloendothelial macrophages in the liver (i.e. Kupffer cells), spleen, and lymph nodes. The remaining iron derived from the breakdown of haeme is subsequently stored as ferritin, or exported by ferroportin into the bloodstream as either NTBI or transferrin. When new erythrocytes are created during erythropoiesis, iron is needed for the haemoglobin molecule. Transferrin facilitates the transportation of stored iron (ferritin) to various tissues throughout the body, including the bone marrow where erythropoiesis takes place. The bulk of iron is taken up by the cells bound to transferrin that enters the cells through endocytosis after binding to the transferrin-receptor (TfR). Each day, 20-25 mg of iron is recycled via the above-explained mechanism of senescent erythrocytes, macrophages, and new erythrocytes (Figure 1.2).

A decade ago, an important hormone regulating these processes of systemic iron homeostasis was finally identified: hepcidin. Hepcidin binds to its receptor ferroportin, and promotes the internal uptake and degradation of ferroportin [23]. Thus, when iron is required, hepcidin expression and production is down-regulated (predominantly) in the liver, enabling ferroportin to export the intracellular iron into the bloodstream. This efflux of stored iron (ferritin) from macrophages predominantly takes place in the liver, the main site for iron storage. Also, more dietary iron residing in the duodenal enterocyte will be released by the up-regulation of ferroportin. Hepcidin expression and production is decreased by erythropoiesis, and hypoxia, and increased by high systemic iron levels and inflammation (Figure 1.2) [24].

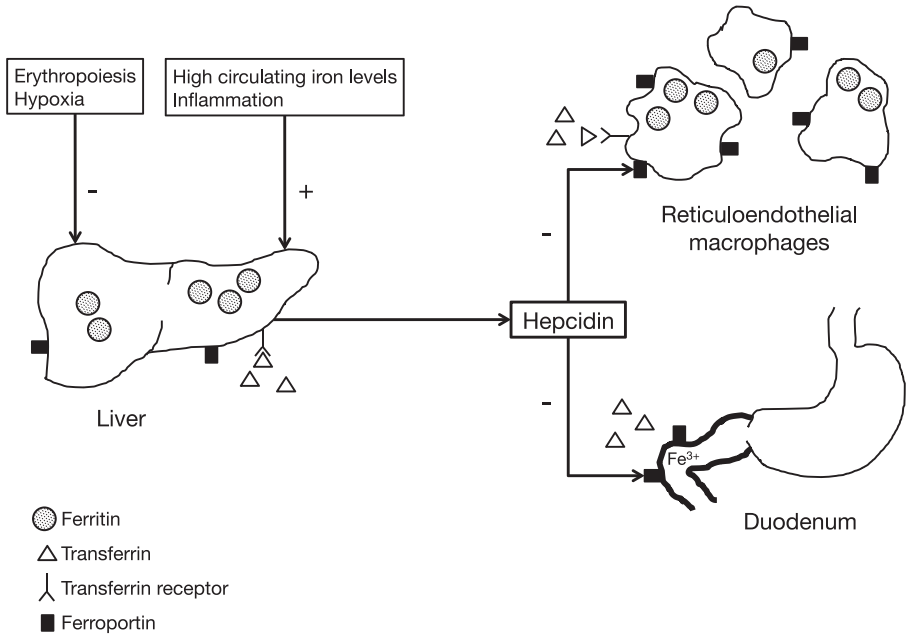


Figure 1.2: Brief outline of iron metabolism regulated by hepcidin. Hepcidin, predominantly produced by the liver, binds to its receptor ferroportin (depicted as black cubes), inducing the internalization and degradation of ferroportin. Ferroportin is the only known cellular iron exporter. High serum hepcidin levels thus block the export of iron into the bloodstream from various storage sites. Iron is exported into the bloodstream safely bound to transferrin (depicted as triangles), and subsequently stored as ferritin (depicted as circles) in liver cells. Reticuloendothelial macrophages derive their iron from the breakdown of senescent erythrocytes which they phagocytose. Increased iron demand, such as in erythropoiesis after blood donation, decreases hepcidin production. Consequently, ferritin can be released from storage sites into the bloodstream and transported to the bone marrow for the production of haemoglobin.

Of note, iron is only dangerous and able to interact when unbound to storage and carrier proteins such as ferritin, transferrin, and haeme. Free, redox-active iron is therefore called "non-transferrin bound iron" (NTBI), which gives rise to the cellular labile iron pool together with chelatable protein-bound iron. Iron is shuttled between different pools of labile iron: the cytosol, mitochondria, and endoplasmic/lysosomal compartment [25]. Under normal conditions, cellular iron homeodynamics is mainly determined by mitochondrial iron consumption [25]. In the mitochondria, ROSs are a naturally occurring by-product of the respiratory chain [26], while at the same place heme-synthesis requires iron. To prevent oxidative cell damage, a delicate balancing system between iron and oxygen exists [25]. The enzymes superoxide dismutase (equilibrating superoxide ($O_2^{\bullet -}$) and peroxide (H_2O_2)) and catalase (subsequently catalysing peroxide (H_2O_2) into water (H_{20}) and oxygen ($\frac{1}{2}$ O_2)) normally serve to remove the (su)peroxide from the cells [27]. Since antioxidants are able to reduce redox stress, dietary antioxidants should decrease the availability of ROSs, but some of them might also serve as pro-oxidants as for example vitamin C (i.e. ascorbate) can replace superoxide in the Haber-Weiss reaction that results in the reactive ferrous iron (Fe^{2+}) [27]. In the cellular cytosol, ferritin also acts as a buffer as it stores Fe^{2+} in the inner cavity after oxidation, a process consuming H_2O_2 [25]. Other factors that increase the Fe^{2+}:Fe^{3+} ratio are hypoxia and inflammatory processes, affecting iron regulatory proteins that bind to iron responsive elements on mRNAs encoding ferritin, ferroportin, TfR, and DMT-1 [25, 28], whereas ferroxidases (such as ceruloplasmin, hephaestin, and zyklopen) could decrease the Fe^{2+}:Fe^{3+} ratio by their ability to oxidize intracellular Fe^{2+} into Fe^{3+}, a necessary step in the process of apotransferrin-bound iron export by ferroportin [22]. In a recent study, the increased iron content in atherosclerotic plaques was confirmed and linked to a possible decrease in ferroxidases, limiting the ability to oxidize ferrous iron into ferric iron [29].

Blood donation is known to lower iron stores [1–3, 30]. Each whole-blood donation of 500 ml contains approximately 200-250 mg iron [3]; a loss that needs to be compensated by increased dietary iron uptake on top of the normal 1-2 mg/day and release from storage sites.

Mechanistic pathways

The exact mechanism of how blood donation would decrease cardiovascular disease remains unclear, but several pathways have ben hypothesised. In Figure 1.3, some of the main hypothesised pathways are set out. It could be caused by a (combination of) the following two pathways: (I) the direct effects of reducing iron subsequently decreasing oxidative stress; (II) a reduction in blood viscosity. Reducing iron reduces oxidative stress, which ameliorates vascular reactivity through increased NO synthesis and action, and decreases the peroxidation of LDL-cholesterol, which in turn is an important progenitor of atherosclerosis [21]. The latter is a process in which macrophages play an important role. They are not only abundant in the vascular intima where atherosclerosis begins, but are also an

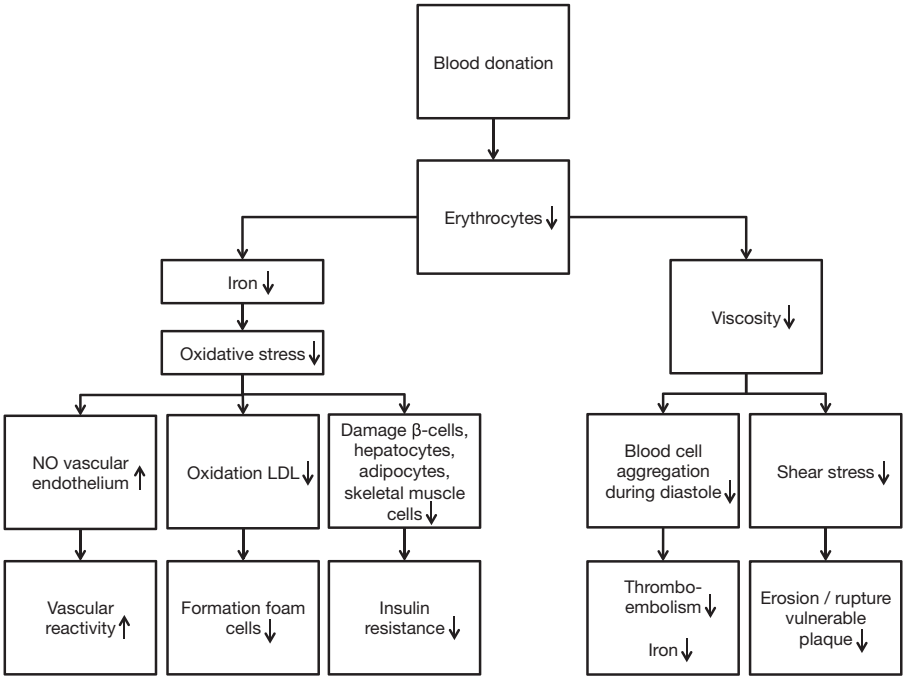


Figure 1.3: Hypothesised pathways of how blood donation reduces cardiovascular disease.

important site for iron storage as they derive their iron from senescent erythrocytes, which they phagocytize. In fact, Sullivan has come up with an update on the iron hypothesis that more specifically describes the importance of macrophages and their iron content in the onset of atherosclerosis [31]. Because of their close proximity to the vascular endothelium, they facilitate the oxidation of LDL-cholesterol which ultimately accumulates into foam cells through a cascade of inflammatory responses [17–19].

Another potentially important pathway yet begins in a completely different physiological compartment: insulin and glucose metabolism. In fact, diabetes mellitus imposes such a huge risk to cardiovascular disease, that it doubles the risk independently from other established risk factors [32]. Of particular interest in this mechanism is the effect of iron on insulin resistance. Insulin resistance can be regarded a pre-diabetic state in which the liver, muscle, and adipose tissue lose their sensitivity to insulin to maintain normal glucose levels. In a range of chronic metabolic conditions, such as dysmetabolic iron overload syndrome, non-alcoholic fatty liver disease, high-ferritin type 2 diabetes mellitus, and metabolic syndrome,

high iron levels seem to accompany insulin resistance and even predict diabetes mellitus [21, 33–35]. The metabolic syndrome is one of the mildest ones in this list of chronic metabolic conditions, but its prevalence is ever-increasing in the western world. It stands for the clustering of several conditions: central obesity, atherogenic lipid profile, hypertension and elevated fasting glucose [36]. As such, it constitutes a risk factor not only for type 2 diabetes mellitus, but also cardiovascular disease.

The mechanisms behind iron and metabolic disturbances are difficult and not yet completely elucidated [21]. First, insulin has long been known to affect the mobilization of transferrin receptors to the cell surface [37]. These receptors facilitate the uptake of transferrin, thereby increasing intracellular iron. Second, iron overload affects insulin resistance through oxidative damage to tissues involved in glucose and lipid metabolism such as pancreatic beta cells, adipose, muscle and also liver tissue [21]. Third, there is an intriguingly and not-yet completely understood interplay between pro-inflammatory molecules, iron, and hormones secreted from adipose tissues [38, 39]. The role of chronic subclinical inflammation has been argued to be the core problem and cause of a disturbed iron homeostasis and its direct and subsequent effects on the atherosclerotic process and insulin resistance.

Importantly, there are other less well-studied hypothesised pathways, among which viscosity [Figure 1.3] [40]. Viscosity can be translated as the thickness and stickiness of blood, and represents more biomechanical forces instead of molecular ones. The removal of erythrocytes with blood donation reduces the viscosity of blood, with less blood cells that normally aggregate at low shear rates such as during diastole, when blood moves slower, to interact with the vascular endothelium [41]. Furthermore, reduced viscosity decreases endothelial wall shear stress that affects the erosion and rupture of vulnerable plaques [41, 42]. There is a small overlap with the "iron reduction pathway", as the decrease in blood cells during diastole also mean a decrease in the amount of iron that is deposited in the thromboembolus from phagocytosed erythrocytes [41].

1.2 Blood Bank Practice in The Netherlands

In the Netherlands, the entire blood supply relies on voluntary non-remunerated donors. Roughly, two different types of blood donors exist: whole-blood donors and plasmapheresis donors. A whole-blood donation consists of the drawing of 500 ml of whole-blood and some additional testing tubes for infectious disease testing purposes. Men are allowed to donate a maximum of 5 donations a year, whereas women are restricted to 3 donations a year. Between each successful donation, at least 56 days must pass by to restore hemoglobin level. Donors that are eligible for donation are invited by postal mail to visit their blood bank within the next two weeks. Approximately 55% of donors respond to such an invitation [43].

After at least 1 successful whole-blood donation, donors are allowed to switch to plasmapheresis. This occurs upon invitation by the donor attendant, and is mainly driven by blood group types, repeated deferrals for malaria-endemic area travels, and repeatedly low Hb levels or when donors experience adverse reactions such as fatigue, dizziness and fainting. Plasmapheresis may occur every two weeks with a maximum of 23 donations per year. Each plasmapheresis procedure collects 650 ml of plasma that is separated from whole-blood in 3 to 4 batches, depending on the machine used. After each separated batch, donors receive their erythrocytes back. The entire collection process takes approximately 45 minutes, whereas a whole-blood donation normally is completed within 10 minutes. Instead of being personally invited by postal mail such as whole-blood donors, plasmapheresis donors often schedule their next appointment after their donation or make/receive a phone call afterwards to make an appointment.

Routinely screening donor health not only ensures donor safety but also the safety of the blood product that is to be derived from the donation. For this purpose, donors have to fill out an eligibility questionnaire during each visit covering lifestyle factors that are related to health status and infectious disease risk. A donor attendant or donor physician evaluates this questionnaire, and subsequently assesses blood pressure, pulse rate and -irregularity, and hemoglobin level by fingerstick method.

1.3 Healthy Donor Effect

Many studies directed to the iron hypothesis have used blood donation as a model for an iron lowering intervention [44–52]. A methodologically challenging aspect is that blood donors are continuously screened and selected on health status throughout their donation career: during the registration process, in-between donations, and before each donation [Figure 1.4]. As a result, blood donors are generally in better health compared to non-donors. Many studies were therefore unable to present unbiased results because of this Healthy Donor Effect (HDE).

Atsma et al. previously described three distinct types of the HDE and referred to them as the Healthy Registration Effect (HRE), the Healthy Donor Survivor Effect (HDSE), and the Healthy Donor Career Effect (HDCE) [53]. When someone applies for blood donorship, a number of health criteria have to be met in order to be registered as a blood donor. This selection process based on (underlying) disease risk is thus responsible for the selection bias or HRE when comparing the health of donors to non-donors.

Once being a newly registered blood donor, donation can only take place if certain health criteria are met. Some of them result in a temporary deferral; others mean the end of a donor's donation career. Consequently, currently active donors are more likely to be healthier than lapsing- or stopped donors. Research comparing these two groups of donors is therefore influenced by the HDSE.

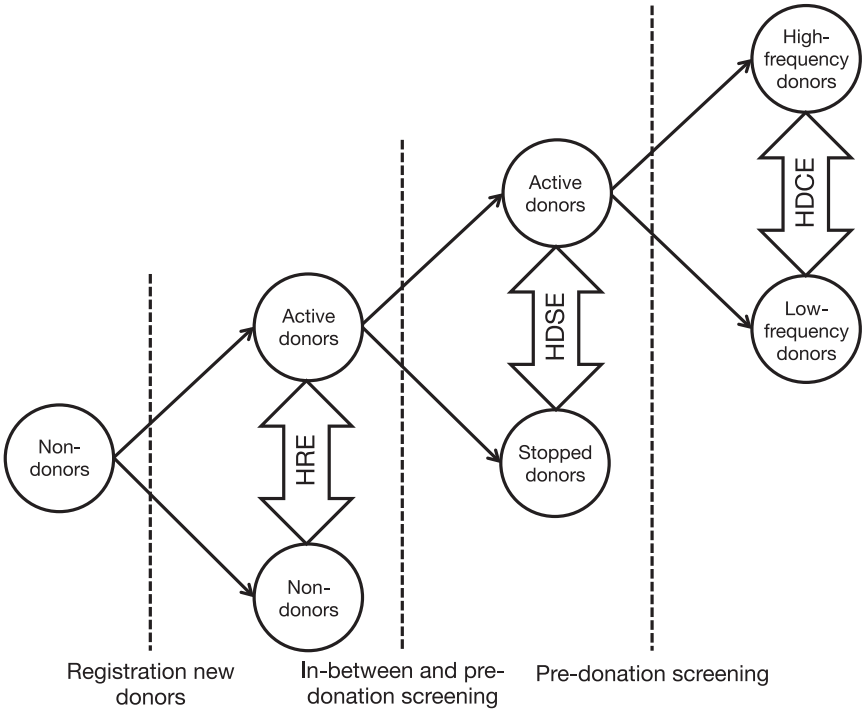


Figure 1.4: Three different types of the HDE (arrows) as a result of three different selection moments (dotted lines) during the donation career. HRE, healthy registration effect; HDSE, healthy donor survivor effect; HDCE, healthy donor career effect.

The third type of HDE, the HDCE, is of importance when studying health effects of blood donation within currently active donors. As a result of continuously applying health criteria prior to each donation, high-frequency donors and those with a higher number of lifetime donations are probably healthier than donors who are yet in an early phase of their donation career.

Concept of Blood Donation

Previous research has shown that the HDE is smaller when making comparisons within the active donor population, as opposed to the general population or stopped donors [53]. However, there are many different aspects about the concept of blood donation as a determinant of exposure, quite similar to that of tobacco use. The "amount" of exposure is determined by three different aspects [Figure 1.5]:

- donation frequency: the number of donations
- donation career: the period during which the donations were made
- the combination of these two aspects: the number of donations per year.

A further combination can be made with the number of donations per year and the donation career as a measure of donation intensity. An average donation frequency per year of 4 is quite intense when the total donation career has been 4 years [see Donor 4 in Figure 1.5], whereas the same donation frequency per year over a donation career of 1.5 years is not [see Donor 1 in Figure 1.5].

When creating contrasts in the spectrum of blood donation, it all comes down to time. The moment of starting, stopping, but also intermittent inactive periods of blood donation determines all of the above three aspects. As such, it is also important to regard the time of blood donation in relation to age. The age at which someone starts donating blood may be an important aspect of the amount and efficacy of blood donation, as it also relates with the progression stage of atherosclerotic disease and the number of years left to continue blood donation.

1.4 Research Objectives

The scope of this thesis is the potential protective effect of lowering iron stores on cardiovascular disease in healthy individuals. As explained above, blood donors constitute an excellent study population to this end, as they are generally iron depleted and disease-free. As a recurring theme throughout this thesis, high-frequency blood donors are compared to low frequency donors in terms of cardiovascular risk, using (lifetime) donation frequency and donation career to create different levels of exposure.

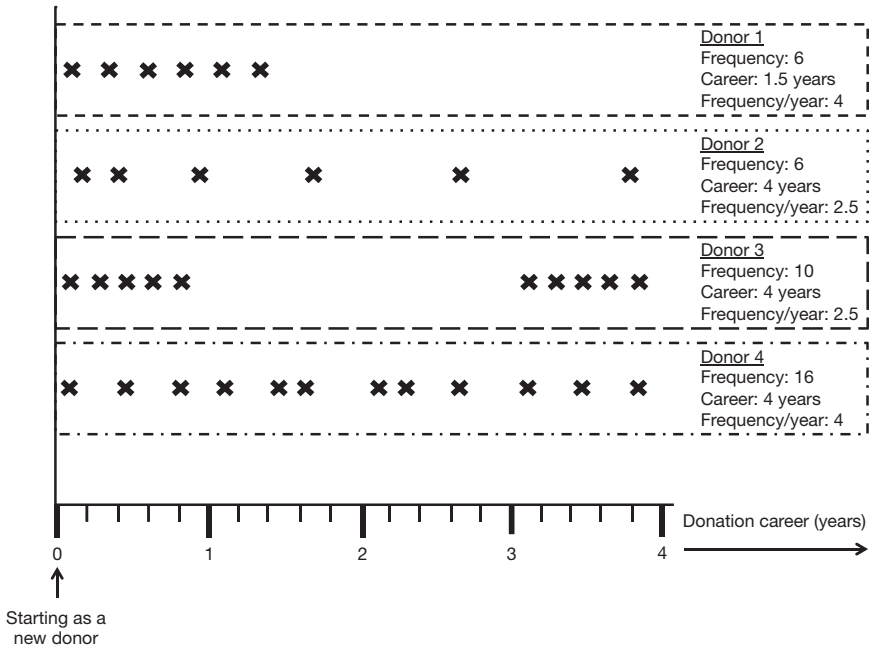


Figure 1.5: Four donors with different donation patterns. Three different aspects determine the exposure to blood donation: donation frequency, donation career, and number of donations per year. Although Donor 1 and Donor 4 have the same average donation frequency per year, Donor 4 is much more exposed as this frequency per year was continued for four years instead of only 1.5 years. To combine donation frequency per year with donation career thus seems a useful measurement of blood donation exposure.

Each donor has his or her own donation career, with a different number of donations and donation frequency per year [Figure 1.5]. This enables us to study possible dose-response relationships between blood donation and cardiovascular disease risk. Furthermore, this thesis explores the use of a qualification period to eliminate the healthy donor effect as much as possible, using a simulation study to test this approach. All with the goal to answer the question whether high-frequency blood donation protects against cardiovascular disease.

1.5 Outline of this Thesis

Developing cardiovascular disease slowly progresses from metabolic changes to subclinical atherosclerosis, until the disease becomes manifest and could ultimately result in cardiovascular death. Essentially, blood donation could interfere with each of these stages in the path to cardiovascular disease. In this thesis, we assess the effect of blood donation on intermediary factors and processes, and also on manifest cardiovascular morbidity and mortality.

Beginning with a cohort of first-time donors, the change in insulin resistance during the first 1.5 - 2 years of the donation career is monitored [Section 2.1]. A more broad outcome measurement of cardiometabolic risk factors (metabolic syndrome) is evaluated next in a larger cohort of currently active whole-blood donors with a longer donation career [Section 2.2]. Continuing to early anatomical changes of the vascular wall, non-invasive measurements of atherosclerosis are investigated in active and stopped blood donors [Section 3.1]. While studying cardiovascular morbidity and mortality, the HDE challenges us again as using such longterm outcomes should not lead to a comparison of active with stopped donors. Furthermore, blood donation should get sufficient opportunity to prevent these longterm effects, requiring a longer period of exposure. In search for a better way to deal with the HDE that also allows for a longer period of blood donation, a simulation study assesses whether the application of a qualification period addresses both issues [Section 4.1]. With this knowledge, the research continues and closes off with overt cardiovascular morbidity and mortality in all Dutch whole blood donors [Section 5.1].

The insights provided by these individual studies will be combined to come to a final conclusion on the debate of the protective ability of blood donation on cardiovascular disease [Chapter 6]. For those who do not have the time to read this entire thesis, an abstract can be found in the Summary on page 137 (for Dutch see the Samenvatting on page 141).

Chapter 2

Cardiometabolic Risk

If frequent blood donation should indeed slow down the onset of cardiovascular disease, then frequent blood can also be expected to be associated with predecessors or risk factors of cardiovascular disease. But what exactly are risk factors of cardiovascular disease? In 1988, 'syndrome X', nowadays called 'metabolic syndrome', was invented by Reaven to describe a cluster of cardiovascular risk factors driven by insulin resistance. Insulin resistance reflects a pre-diabetic stage. Metabolic syndrome is a cluster of 5 traits that constitutes a huge risk for both cardiovascular disease and type 2 diabetes mellitus.

In this Chapter, we will describe whether frequent blood donation is associated with an improved insulin resistance and lower prevalence of metabolic syndrome. We will start with a cohort of first-time blood donors, in which the change in insulin resistance is determined over a course of 1.5 - 2 years after the very first blood donation. This may, however, not be long enough to detect an improvement of insulin resistance. Currently active whole-blood donors do have a longer donation history. Therefore, a study that determines the presence of metabolic syndrome among currently active blood donors is described next.

2.1 Insulin Resistance

Original manuscript title:
Blood donation and insulin resistance in a follow-up study of first-time donors.
K. Peffer, M. den Heijer, D. W. Swinkels, A. J. Geurts-Moespot, F. Atsma, A. L. M. Verbeek
Submitted for publication

BACKGROUND: Iron depletion could improve insulin resistance by lowering oxidative stress in hepatocytes, adipose tissue and pancreatic β-cells. Blood donation is effective in depleting iron stores. This study aims to test whether frequent whole-blood donation is able to improve insulin resistance in a cohort of first-time blood donors.
STUDY DESIGN AND METHODS: A cohort of male and female first-time whole-blood donors $\geq$ 45 years, was followed-up for 1.5 - 2 years. Insulin resistance (HOMA2-IR) and other cardiometabolic risk factors were measured after overnight fasting at baseline (=before 1st blood donation) and follow-up (median follow-up time = 20 months). Individual percentage changes from baseline were calculated with 95% confidence intervals (CIs).
RESULTS: Of 120 first-time donors at baseline, 112 participated at follow-up. Ferritin and hepcidin strongly decreased, both showing a dose-response effect with number of donations (p for trend <0.001). HOMA2-IR increased during follow-up, by 5.7% (95%-CI: -8.4 to 22.0) in men and 23.9% (95%-CI: 9.5 to 40.1) in women. This increase was not explained by number of donations during follow-up (p for trend=0.392), or by changing alanine aminotransferase levels (r=0.074, p=0.219), but was positively associated with follow-up time (r=0.172, p=0.036).
CONCLUSION: Although iron stores were greatly reduced by blood donations in this cohort of first-time donors, insulin resistance increased instead of decreased, especially in women. The observed increase in insulin resistance during follow-up is best explained by ageing. Blood donation does not improve insulin resistance in middle-aged healthy subjects.

Introduction

Increased iron stores and metabolic disturbances go hand in hand. In patients with unexplained hepatic iron overload, the prevalence of the insulin resistance syndrome, a predecessor of the metabolic syndrome (MetS), is 94% [54]. The other way around: the prevalence of excess body iron is 15% in patients with MetS [55]. Furthermore, there is a considerable association between these two disturbances and the presence of hepatic steatosis known as non-alcoholic fatty liver disease (NAFLD): 42% of NAFLD-patients has MetS [56]. This overlap between mild hepatic iron overload, insulin resistance, and hepatic steatosis accompanied by transferrin saturation in the upper-normal range is now recognized as the dysmetabolic iron overload syndrome (DIOS) and constitutes a risk marker for type 2 diabetes mellitus (T2DM) and cardiovascular disease [33, 34, 57, 58].

The underlying pathogenic pathway is not yet completely understood, but subclinical inflammation and disturbances in iron homeostasis are probably of key importance [33, 34]. Inflammatory processes increase hepatic hepcidin expression, the key iron regulatory hormone [23, 59, 60]. In a mouse model, starvation-induced gluconeogenesis has also been found to increase hepatic hepcidin expression [61]. As a consequence, iron accumulates in the reticuloendothelial system, as hepcidin is responsible for the internalization and subsequent degradation of ferroportin, the only known cellular iron exporter. Increased body iron could exert its effect on insulin resistance by interfering with insulin receptor signaling and expression and inhibiting the ability to burn carbohydrates in the liver and muscle [33, 34]. Iron is a known catalyzer of the formation of reactive oxygen species (ROSs). The insulin receptor expression and insulin signaling of liver cells and peripheral tissue can be hampered by ROSs, thus contributing to insulin resistance [62]. Moreover, iron has been observed to compromise the functioning of adipose tissue, resulting in adipocyte insulin resistance and hypertriglyceridemia [38, 39]. Therefore, increased iron stores could be responsible for cell damage, which can result in liver damage, insulin resistance, β-cell dysfunction, and ultimately diabetes mellitus. Increased baseline iron stores have already been found to predict MetS and T2DM [63–66]. This raises the question whether iron depletion can prevent the development of insulin resistance, MetS, and related conditions such as NAFLD, DIOS, and T2DM.

Iron depletion occurs by reducing iron stores. Excess iron is stored in the liver and the reticuloendothelial macrophages in the form of ferritin. Since serum ferritin levels reflect the levels of tissue iron stored in ferritin, serum ferritin is a biomarker of (macrophage) iron status [67]. The key regulatory hormone of iron homeostasis is hepcidin, which regulates intestinal iron absorption and iron efflux from the storage sites. Thus, plasma hepcidin is a good biomarker of systemic iron homeostasis [68].

Blood donation is an effective intervention to deplete iron stores. Although Hb levels are tightly monitored in order to prevent anaemia, serum ferritin and hepcidin levels are diminished two-fold by frequent blood donation [69]. Thus, blood donors constitute a healthy, naturally iron-depleted population in which such a question can be studied. There have been several studies that have confirmed a protective effect of frequent blood donation on insulin

resistance and MetS [48, 70–72], but some have proven otherwise [50, 73]. We recently found that high intensity blood donation was associated with increased prevalence of MetS in non-obese women, but not in obese women or in men [74].

The difficulty in making causality claims from these aforementioned studies is the absence of follow-up data. Due to the fact that these studies were all cross-sectional in design, no temporal relationship between lowering iron stores and metabolic alterations could be studied. Therefore, we have conducted a longitudinal study among first-time blood donors in which cardiometabolic risk factors were measured at baseline and 1.5 - 2 years thereafter. The aim of this study is to test whether blood donation is able to improve insulin resistance, and whether changes in insulin resistance relate with changing iron parameters in healthy blood donors.

Materials and Methods

Study population

In two large blood bank collection centers in the east of the Netherlands, all first-time whole-blood donors $\geq$ 45 years were invited by postal mail to participate in the CARdiovascular risk and DONation (CARDON)-study. In the Netherlands, first-time donors are newly registered whole-blood donors who have successfully passed their first medical screening and are eligible to become a blood donor. This includes the absence of insulin-dependent T2DM. Between December 2010 and January 2012, 175 first-time donors were invited to participate (99 women and 76 men). During the visit for the CARDON-study, they were given the opportunity to make their first whole-blood donation. A total of 161 (92.0%) responded to the invitation, of which 131 (74.9%) were willing to participate and eventually 120 (68.6%) participated.

After a mean period of 20 months, all 120 donors were re-invited to participate in follow-up measurements. Between January and May 2013, 112 donors showed up for their follow-up measurements. They included both active and stopped donors. Aimed at 80 donors at follow-up measurement, this study had a power of 90% to detect a difference of 0.35 in HOMA-IR between baseline and follow-up measurement with an alpha of 0.05 (one-sided). All participants provided written informed consent after full explanation of the purpose and nature of all study procedures. The accredited Medical Research Ethics Committee Region Arnhem-Nijmegen approved this study's protocol, which is in accordance to the Declaration of Helsinki on ethical principles for medical research involving human subjects.

Data collection

Visits were scheduled between 8:00 and 10:30 am. Donors were sent a questionnaire by mail, which they were suggested to fill in at home and bring with them to the visit. The questionnaire covered topics such as lifestyle factors, disease history, familial history of cardiovascular disease, and reproductive factors of women. All participants were instructed

by phone and postal mail to abstain from smoking and to fast at least 8 hours before the study visit. The research assistants or the researcher reviewed all answers during the visit, and inconsistencies were discussed with the donor. Anthropometric measurements were performed according to standardized protocols and conducted in duplo, consisting of: blood pressure, waist- and hip circumference, height and weight. Waist circumference was measured with a soft tape at the level midway between the lowest rib and iliac crest. The hip circumference was measured at the level of the head of the femur. Measurements started and ended with blood pressure measured manually (OMRON Digital Blood Pressure Monitor, HEM-907 Intellisense, and WelchAllyn, Maxi Stabil 3) at the right arm in sitting position. Shoes, jackets/coats and heavy clothing were taken off during all measurements. Circumferences were adjusted by minus 2π times thickness of clothing. BMI (kg/m^2) was calculated by dividing weight in kilograms by squared height in meters. Donors were instructed to bring the packaging of their medication used within 2 weeks before the visit with them.

Finally, fasting venous blood samples were drawn. Glucose and alanine aminotransferase (ALT) were both measured in plasma using Abbott reagents on the ARCHITECT C16000 (Abbott BV Diagnostics Division, Hoofddorp, The Netherlands). Insulin was determined in plasma with a two-step electrochemiluminescence immunoassay using a test kit from Roche Diagnostics on the Modular E170 (Roche/Hitachi Modular Analytics E170, Basel, Switzerland). Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, ferritin, transferrin, iron, and high-sensitivity c-reactive protein (hs-CRP) were measured on an Olympus AU 400 (Beckman Coulter, Switzerland). Hb and mean corpuscular volume (MCV) were determined by on the SysmexXT1800i (Sysmex Corporation, Kobe, Japan). Zinc protoporphyrin (ZPP) was measured on a ZPP Hematofluorometer (Aviv Biomedical, Inc., Lakewood NJ USA). Hepcidin was measured in Li-heparin plasma with an in-house competitive ELISA as described previously [68, 75]. Some subjects had plasma hepcidin levels below the lower limit of detection (LLOD, 0.2 nmol/L) at a 20-fold dilution. Those subjects with levels at baseline below the LLOD (n=6) were imputed a value that was randomly drawn from a uniform distribution with minimum 0 and maximum 0.2 nmol/L. At follow-up, all donors (n=15) with initial values below 0.8 nmol/L were re-determined with a 5-fold dilution. In this rerun, the LLOD was 0.17 nmol/L. The values from the rerun were used in the analysis.

Homeostasis model assessment-insulin resistance (HOMA-IR) There are several models that estimate insulin resistance. HOMA-IR has been found to strongly correlate with estimates obtained by hyperinsulinemic euglycemic clamp and is one of the recommended models to be used in large epidemiologic studies [76, 77]. We have used the updated, more precise, computerized version, HOMA2 [78]. Higher HOMA2-IR scores indicate increasing insulin resistance compared to the reference population in which it was developed.

Number of donations All whole-blood donations that occurred during follow-up and had a drawn volume of at least 100 ml were cumulated. In the Netherlands, donors are allowed to donate 3 (women) or 5 (men) times a year with minimum intervals of 56 days. Also, donors must meet the criteria of $\geq 50 \leq 100$ mmHg diastolic and $\geq 90 \leq 180$ mmHg systolic blood pressure, and a capillary Hb level between $\geq 7.8 \leq 11.0$ mmol/l for women and $\geq 8.4 \leq 12.0$ mmol/l for men before donation.

Statistical analysis

Because HOMA2-IR follows a right-skewed distribution, log_e-transformation was applied to retrieve geometric means of baseline and follow-up measurements. This was also calculated for triglycerides, cholesterol, TC/HDL ratio, ALT, total iron, TSAT, ferritin, hepcidin and the hepcidin/ferritin ratio. Individual percentage change from baseline was calculated for normally distributed descriptive variables to obtain mean percentage change and its corresponding 95%-CI. Likewise, individual change in ln-transformed variables was calculated to obtain the mean percentage change and its corresponding 95%-CI via back-transformation:

$$(e^{\frac{1}{n}\sum_{i=1}^{n}(log(x_i)_{follow-up} - log(x_i)_{baseline})} - 1) * 100\%.$$

Change in HOMA2-IR, ferritin, hepcidin, and ALT was calculated by subtracting individual baseline values from follow-up values. Linear non-parametric trend analyses were performed for change in ferritin, hepcidin, and HOMA2-IR across number of whole-blood donations using Jonckheere-Terpstra's one-sided test. Spearman's correlation coefficient with a one-sided p-value was calculated to assess the association between change in ALT and change in HOMA2-IR, as well as for the association between follow-up time and change in HOMA2-IR.

All analyses were performed using IBM SPSS Statistics 21, Release Version 21.0.0.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Mac, Version 21.0. Armonk, NY: IBM Corp.).

Results

More women (n=65, 54.2%) participated at baseline than men (n=55, 45.8%). Of them, 61 women and 51 men also participated in the follow-up measurement, at which time 97 (86.6%) donors were still active. Median follow-up time was 20 months, during which a range of 0-9 donations was made, with a median number of 3 donations.

ALT increased whereas triglycerides, ferritin, and hepcidin all significantly decreased during follow-up [Table 2.1]. In men, ferritin decreased on average with -59.9% (95%-CI: -66.7 to -51.7) and hepcidin decreased with -31.3% (95%-CI: -51.0 to -3.6). The hepcidin/ferritin ratio, reflecting the appropriateness of the decrease in hepcidin relative to ferritin, increases with +71.4% (95%-CI: 29.7 to 126.6). Women show similar, but less pronounced, patterns in iron homeostasis during follow-up [Table 2.1].

Table 2.1: Characteristics of study population (n=112) at baseline and follow-up measurements

	Men n=51			Women n=61		
Characteristic	**Baseline** Mean (sd)	**Follow-up** Mean (sd)	Mean individual change (95%-CI)	**Baseline** Mean (sd)	**Follow-up** Mean (sd)	Mean individual change (95%-CI)
Age (yr)	52.2 (5.6)	53.9 (5.5)	+3.3% (3.0 to 3.6)	52.3 (4.8)	53.9 (4.0)	+3.2% (2.9 to 3.4)
BMI (kg/m^2)	26.9 (4.0)	26.9 (4.1)	-0.1% (-1.5 to 1.3)	25.7 (4.0)	26.1 (4.4)	+1.5% (-0.2 to 3.2)
Waist (cm)	98.2 (11.4)	97.6 (11.3)	-0.5% (-1.9 to 0.9)	88.8 (10.5)	90.1 (11.8)	+1.6% (-0.2 to 3.4)
Waist-to-hip (cm)	0.98 (0.07)	0.98 (0.06)	+0.2% (-1.2 to 1.7)	0.87 (0.07)	0.89 (0.07)	+1.7% (-0.2 to 3.5)
Triglycerides[a] (mmol/l)	1.48 (1.66)	1.26 (1.67)	-14.9% (-23.4 to -5.5)	1.07 (1.53)	1.07 (1.68)	-0.3% (-9.3 to 9.6)
Cholesterol[a] (mmol/l)	5.31 (1.57)	5.54 (1.23)	+4.4% (-7.2 to 17.4)	5.65 (1.18)	5.56 (1.17)	-1.6% (-4.8 to 1.6)
HDL-c (mmol/l)	1.34 (0.31)	1.32 (0.30)	+0.2% (-3.7 to 4.1)	1.59 (0.37)	1.57 (0.40)	-0.8% (-4.5 to 2.8)
LDL-c (mmol/l)	4.35 (0.96)	4.15 (0.97)	-2.3% (-8.7 to 4.1)	4.14 (0.88)	4.01 (0.79)	-2.0% (-5.4 to 1.4)
TC/HDL ratio [a]	4.08 (1.58)	4.29 (1.30)	+5.2% (-6.7 to 18.5)	3.64 (1.27)	3.65 (1.28)	+0.2% (-3.7 to 4.3)
ALT[a] (U/l)	28 (1)	31 (1)	+10.6% (-1.2 to 23.8)	21 (2)	28 (1)	+34.8% (24.4 to 46.1)
Hs-CRP[a] (mg/l)	1.11 (2.41)	1.12 (2.95)	+0.8% (-19.9 to 26.8)	1.50 (2.89)	1.49 (2.41)	-0.8% (-16.8 to 18.2)
Deferrals for (N(%))						
Low Hb						
0 deferrals	51 (100)	38 (74.5)		56 (91.8)	50 (82.0)	
1 deferral	0 (0.0)	9 (17.9)	N/A	2 (3.3)	8 (13.1)	N/A
2 deferrals	0 (0.0)	2 (3.9)		2 (3.3)	2 (3.3)	
3 deferrals	0 (0.0)	2 (3.9)		1 (1.6)	1 (1.6)	
Blood pressure						
0 deferrals	51 (100)	50 (98.0)		60 (98.4)	59 (96.7)	
1 deferral	0 (0.0)	1 (2.0)	N/A	0 (0.0)	1 (1.6)	N/A
2 deferrals	0 (0.0)	0 (0.0)		1 (1.6)	0 (0.0)	
3 deferrals	0 (0.0)	0 (0.0)		0 (0.0)	1 (1.6)	

TC/HDL ratio; total cholesterol/HDL ratio; hs-CRP, high-sensitivity c-reactive protein; MCV, Mean Corpuscular Volume; ZPP, Zinc Protoporphyrine; TSAT%, Transferrin saturation calculated by (100xtotal iron) /(transferrinx25); HOMA2-IR, Homeostasis model assessment 2 - insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; N/A, Not Applicable. [a] Geometric mean (sd) derived from natural logarithm transformation. Mean percentage change is derived from individual changes from baseline.

Table 2.1: Characteristics of study population (n=112) at baseline and follow-up measurements (continued)

	Men n=51			Women n=61		
Characteristic	**Baseline** Mean (sd)	**Follow-up** Mean (sd)	Mean individual change (95%-CI)	**Baseline** Mean (sd)	**Follow-up** Mean (sd)	Mean individual change (95%-CI)
Iron parameters						
Haemoglobin (mmol/l)	9.5 (0.5)	9.3 (0.6)	-1.5% (-2.9 to -0.1)	8.7 (0.4)	8.5 (0.5)	-1.8% (-3.1 to -0.6)
MCV (fl)	88.2 (4.3)	88.7 (4.8)	+0.6% (-0.3 to 1.4)	88.3 (3.4)	88.2 (4.2)	-0.1% (-0.8 to 0.6)
ZPP (μmol/mol heme)	49 (15)	54 (20)	+10.9% (3.7 to 18.0)	61 (22)	66 (25)	+12.2% (3.7 to 20.7)
Total iron[a] (μmol/l)	18.8 (1.4)	17.4 (2.20)	-7.4% (-18.1 to 4.6)	17.0 (1.5)	15.5 (1.5)	-9.2% (-18.8 to 1.6)
Transferrin (g/l)	2.46 (0.34)	2.55 (0.39)	+6.4% (-2.5 to 15.2)	2.67 (0.42)	2.64 (0.45)	-1.0% (-4.2 to 2.2)
TSAT[a] (%)	31.0 (1.3)	27.6 (1.6)	-10.9% (-22.0 to 1.8)	25.8 (1.6)	23.7 (1.6)	-7.4% (-18.0 to 4.6)
Ferritin[a] (μg/l)	158.3 (2.0)	63.4 (2.2)	-59.9% (-66.7 to -51.7)	66.1 (2.0)	35.8 (2.1)	-45.8% (-54.6 to -35.3)
Hepcidin[a] (nmol/l)	4.8 (2.4)	3.3 (2.6)	-31.3% (-51.0 to -3.6)	2.9 (3.9)	2.4 (2.6)	-18.2% (-39.8 to 11.2)
Hepcidin/ferritin[a] (μmoles/μg)	30.3 (2.8)	51.9 (1.9)	+71.4% (29.7 to 126.6)	44.5 (2.7)	67.1 (1.8)	+50.9% (19.6 to 90.5)
Outcome measurements						
HOMA2-IR[a] (mmol/l)	1.17 (1.97)	1.24 (2.03)	+5.7% (-8.4 to 22.0)	1.06 (1.74)	1.32 (1.81)	+23.9% (9.5 to 40.1)
Glucose (mmol/l)	5.25 (1.09)	5.24 (1.13)	-0.3% (-3.3 to 2.7)	5.02 (1.12)	5.08 (1.15)	+1.0% (-1.1 to 3.2)
Insulin (mU/l)	7.76 (1.96)	8.28 (2.05)	+6.7% (-7.9 to 23.6)	7.14 (1.74)	8.87 (1.79)	+24.2% (9.9 to 40.3)
SBP (mmHg)	131 (15)	132 (13)	+0.9% (-1.1 to 2.9)	128 (15)	127 (15)	-0.6% (-3.0 to 1.9)
DBP (mmHg)	85 (10)	85 (9)	+0.2% (-2.1 to 2.5)	82 (9)	81 (8)	-0.6% (-2.9 to 1.7)

TC/HDL ratio; total cholesterol/HDL ratio; hs-CRP, high-sensitivity c-reactive protein; MCV, Mean Corpuscular Volume; ZPP, Zinc Protoporphyrine; TSAT%, Transferrin saturation calculated by (100xtotal iron) /(transferrinx25); HOMA2-IR, Homeostasis model assessment 2 - insulin resistance; SBP, systolic blood pressure; DBP, diastolic blood pressure; N/A, Not Applicable. [a] Geometric mean (sd) derived from natural logarithm transformation. Mean percentage change is derived from individual changes from baseline.

Change in ferritin [Figure 2.1a] and hepcidin [Figure 2.1b] both showed a clear dose-response relationship with number of donations during follow-up (p for trend <0.001 for both ferritin and hepcidin).

Men had a slight increase in HOMA2-IR during the study period, from a (geometric) mean of 1.17 at baseline to 1.24 at follow-up [Table 2.1]: an increase of 5.7% (95%-CI from -8.4 to 22.0). However, women showed a significant increase in HOMA2-IR of +23.9% (95%-CI: 9.5 to 40.1) during the study period follow-up.

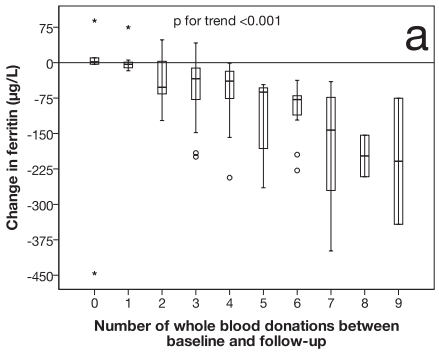
Overall, no consistent dose-response relation was observed for number of donations and change in HOMA2-IR [p for trend=0.392, Figure 2.1c]. As HOMA2-IR actually deteriorated during follow-up instead of improved, no further attempts were made to relate changes in iron parameters to changes in HOMA2-IR. Because ALT, a marker of liver damage, increased during follow-up, we assessed its association with change in HOMA2-IR (r=0.074, p=0.219) and confirmed that any deterioration in liver function did not explain the observed increase in insulin resistance [Figure 2.2]. However, follow-up time was positively associated with change in HOMA2-IR (r=0.172, p=0.036), possibly reflecting an ageing effect.

Discussion

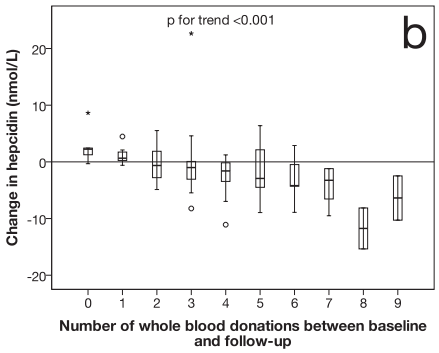
In a cohort of first-time whole-blood donors, the course of 1.5 years of donating blood was accompanied by a strong decrease in ferritin and hepcidin, whereas insulin resistance increased with 23.9% in women. This unexpected increase in insulin resistance was not explained by number of blood donations. These findings show that blood donation depletes iron stores, but does not improve insulin resistance.

There are three explanations for our results: 1) a protective effect of blood donation on insulin resistance does not exist; 2) a protective effect of blood donation is masked by an non-specific time effect, meaning that non-donors would have deteriorated much worse in insulin resistance than donors; 3) the exposure window was too short to have exposed a protective effect, i.e. only acute effects on iron were visible while it takes longer to find the subsequent beneficial effects on insulin resistance. The latter is somewhat reasonable as our donors were generally healthy with insulin resistance already quite low, making further improvement hard to prove. Nevertheless, iron stores were depleted, as ferritin and even the regulatory hormone hepcidin both strongly decreased. Since the few donors that did not donate at all during follow-up could be regarded as a control group, but showed no more increase in insulin resistance than those with >0 donations [Figure 2.1c], the second explanation seems somewhat unlikely.

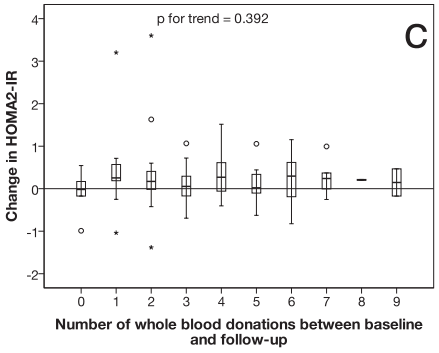
This is the first study to investigate the longitudinal effect of multiple blood donations in a cohort of first-time blood donors on insulin resistance and other cardiometabolic parameters. In a recent study, forty-two men with normal glucose tolerance were followed for three weeks, of whom 10 were studied for nearly four months. After one 450ml donation on t0, insulin resistance (HOMA-IR) unexpectedly increased with 5.9% from a mean of 2.55 (±0.16) at baseline to 2.75 (±0.46) at the final visit nearly four months later [73].



(a) Change in ferritin (μg/L) by number of donations



(b) Change in hepcidin (nmol/L) by number of donations



(c) Change in HOMA2-IR (mmol/L) by number of donations

Figure 2.1: Boxes depict medians, 25th, and 75th percentiles. Whiskers are the reasonable minimum and maximum values (disregarding outliers and extremes). Circles are outliers (>1.5 and <3 times IQR from box's end); asterisks are extremes (>3 times IQR from box's end). All change scores are calculated by subtracting baseline values from follow-up values. P-values for a descending trend of medians are one-sided and derived from Jonckheere-Terpstra's test.

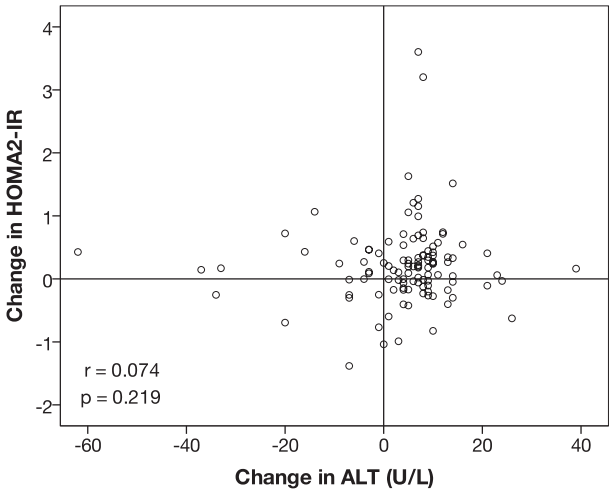


Figure 2.2: Scatterplot of change in HOMA2-IR by change in ALT (U/L), assessing the association between the two variables by Spearman's correlation coefficient and its one-sided p-value.

Other studies have been mainly cross-sectional in design. This could explain why Fernández-Real and colleagues have found high-frequency donors to have an improved insulin sensitivity compared to low-frequency donors; the same observation that Zheng et al. have made albeit a non-statistically significant one [48, 50]. In such cross-sectional designs, the healthy donor survivor effect could have been part of the explanation for the results [53]. Those donors who were able to have donated that often must have been healthy enough to do so. However, a study that was simultaneously performed with the current study by our research group, also using a cross-sectional design comparing low- and high-intensity donors, no sign of a healthy donor survivor effect was observed. Surprisingly, although only described in the population characteristics of our previous study, insulin resistance was positively associated with donation intensity [74]. Since these active whole-blood donors stem from the same source population as the first-time donors in the current study, it seems plausible that we have observed the same pattern in the current study.

Furthermore, the age at which someone starts donating blood could have influenced the results in this study; there still needs to be something to be averted by blood donation. The first-time donors in our study were all at least 45 years at inclusion. To this point, our donors might have been too late (or old) to improve insulin resistance. Of note, our donors were already quite healthy with regard to insulin resistance, making further improvement difficult to detect. Actually, this was also the reason why we have specifically included middle-aged donors, as to assure that differences in cardiometabolic risk could be detected at all.

On the other hand, it could be the very reason why our donors, mainly women, showed a worsening in insulin resistance, merely reflecting the process of ageing that occurred during follow-up. The positive association between follow-up time and change in HOMA2-IR also supports this idea. The finding that the deterioration in insulin resistance was much larger in women, mainly driven by an increase in insulin, points to hormonal changes as a result of menopause that is known to increase at least skeletal muscle insulin resistance but possibly also hepatic insulin resistance [13].

One other longitudinal prospective study found such deteriorating insulin sensitivity in Mexican-American women during 4 years of follow-up [79]. The decrease in our middle-aged women of 19.7% (calculated backwards from IR to HOMA-%S, as insulin sensitivity is the reciprocal of IR) approaches the one observed in Mexican-American women, which was 15.7%. However, two other longitudinal prospective studies with yet completely different subjects found decreases in insulin resistance of 60% one year after bariatric surgery [80] and 14.3% in young adults (freshmen) during 4 years of follow-up [81].

Reducing insulin resistance has gained importance now that it has been found to be an independent predictor of cardiovascular events in the general population [82–86]. Although phlebotomy could still be a powerful intervention to lower iron stores in patients with DIOS, MetS, NAFLD, and perhaps even T2DM, this study could not confirm its preventive potential in a cohort of healthy first-time donors on insulin resistance. However, a recent randomized controlled trial in patients with NAFLD could also not confirm improved insulin resistance after phlebotomy [87]. Of note, in diseased individuals, phlebotomy could have quite different effects than in a healthy donor population, as inflammatory responses accompanying metabolic conditions affect iron metabolism. Therefore, the current study was specifically designed to test its preventive potential on cardiometabolic risk in healthy subjects.

Besides the iron-hypothesis, which includes the much-researched pathway of iron accumulation in intimal macrophages and subsequent atherosclerosis, other pathophysiological mechanisms of cardiometabolic risk reduction through phlebotomy are relatively unexplored. For example, it has also been proposed that whole-blood donation reduces viscosity [41] as a result of lowering haematocrit. In turn, this could reduce vascular endothelial wall shear stress, thus avoiding rupture and erosion of vulnerable plaques.

In conclusion, blood donation decreases iron stores in first-time donors after 1.5 - 2 years of follow-up, but this was not accompanied by an improved insulin resistance. Overall, donors had a deteriorating insulin resistance, probably due to ageing. If blood donation would lower cardiovascular disease risk, it seems unlikely that insulin resistance is involved.

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Competing interests

The authors have no competing interests.

Author contributions

K.P. collected, interpreted, and analyzed the data, wrote, reviewed, and edited the manuscript. F.A. co-ordinated the study, contributed to study design, interpreted the data, contributed to discussion, and reviewed the manuscript, A.L.M.V. and M.H. contributed to study design, interpreted the data, contributed to discussion, and reviewed the manuscript, D.W.S. contributed to study design and reviewed the manuscript, A.J.G.-M. provided assistance with data collection. K.P. and F.A. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

2.2 Metabolic Syndrome

Original manuscript title:
Donation intensity and metabolic syndrome in active whole-blood donors.
K. Peffer, A. L. M. Verbeek, D. W. Swinkels, A. J. Geurts-Moespot, M. den Heijer, F. Atsma
Vox Sanguinis 2015, 109(1), pp. 25-34.

BACKGROUND AND OBJECTIVES: Increased iron and metabolic syndrome (MetS) go hand in hand. Frequent blood donation depletes iron stores. This study investigates whether high-intensity blood donation is associated with lower MetS prevalence compared with low-intensity blood donation, and whether iron acts as an intermediary factor.
MATERIALS AND METHODS: A random sample of 422 male and 211 female active whole-blood donors ≥ 45 years of age was included in a cross-sectional study. Lipids, glucose and iron parameters were measured after overnight fasting. MetS was defined according to the joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention. Three groups of donation intensity were created by sex-specific tertiles of donation frequency per year and duration of donor career.
RESULTS: MetS was present in 22.9% of donors. Prevalence of MetS was 1.46 (95%-Confidence Interval [CI]: 0.93 to 2.30) times higher in men with high donation intensity, whereas in women MetS prevalence was 2.14 (95%-CI: 0.94 to 4.86) times higher in donors with high donation intensity compared with those with low donation intensity. In men, increased prevalence of MetS was mainly associated with higher ferritin, whereas high hepcidin predominantly affected MetS prevalence in women.
CONCLUSION: High-intensity blood donation is not associated with a decreased prevalence of MetS. In men and women, different iron parameters are associated with MetS prevalence. The temporal relationship between blood donation, iron, and MetS, and gender differences herein need to be explored in future research.

Introduction

Ever since the iron hypothesis of cardiovascular disease was proposed in 1981 [8], studies investigating associations between iron stores and cardiovascular risk have had conflicting results. Originally, the hypothesis was brought up by Sullivan to synthesize several observations: 1) increased CVD risk in western civilizations compared with developing countries, where iron deficiency occurs more often due to malnutrition; 2) myocardial failure in iron overload disease; 3) increased CVD risk in postmenopausal (non-menstruating) women compared with premenopausal women accompanied by increasing iron levels in postmenopausal women, and a lifetime increase of iron levels in men, which would explain the established difference in cardiovascular risk between men and women [8]. Cardiovascular disease and its (metabolic) risk factors, such as hypertension, atherogenic dyslipidaemia, abdominal obesity, and insulin resistance, often referred to as the Metabolic Syndrome (MetS), are increasingly prevalent in European countries, with prevalences of MetS of 34.0% in men and 24.1% in women in the Netherlands in 2009 and 2010 [88]. MetS is a strong predictor of cardiovascular disease and type 2 diabetes (T2DM) [89, 90].

As to the mechanism of action, there is firm evidence that inflammation and (subsequent) excess iron contribute to oxidative stress and endoplasmic reticulum stress and thus potentially to the pathogenesis of insulin resistance and atherosclerosis [31, 58]. Increased iron stores have been repeatedly shown to be associated with the metabolic syndrome, but the causal sequence and underlying pathways remain poorly understood [33, 34]. Increased serum ferritin has been associated with onset of MetS and T2DM [63, 65, 91], possibly by affecting insulin receptor expression/affinity and insulin signalling [62]. On the other hand, insulin is known to influence iron metabolism by stimulating iron uptake in adipocytes and hepatocytes [37]. Also, the key regulatory hormone of iron metabolism, hepcidin, was recently found to be up-regulated by gluconeogenesis [61], and also by oral glucose loading in healthy subjects [92]. Moreover, hormones secreted from adipose tissue, such as adiponectin en leptin, have been found to interact with iron metabolism as well [39, 93, 94].

Sullivan has suggested that to test the iron-cardiovascular disease hypothesis accurately, subjects should have depleted iron stores but normal Hb levels [95], which can be obtained by recurrent phlebotomy [1, 2]. To date, two RCTs have been conducted that tested the iron hypothesis in selected patient groups by randomly assigning them to either phlebotomy or a (waiting-list) control group. The FeAST-study included patients suffering from peripheral arterial disease, which showed no effect of randomly assigned 6-month interval phlebotomy on all-cause mortality or death plus MI and stroke [96]. In a second RCT, patients with metabolic syndrome had an improved systolic blood pressure at 6 weeks follow-up after two phlebotomies compared with a waiting-list control group [97]. No significant changes were observed for insulin sensitivity. Observational studies, however, did find iron depletion to ameliorate insulin resistance in patients with non-alcoholic fatty liver disease, the hepatic manifestation of MetS [62, 72].

Because these previous studies were mainly performed in clinical populations, reverse causation is a possible explanation of the observed effect. Therefore, additional research has been performed in blood donors, as blood donors are a generally healthy population and the regular loss of whole-blood leads to decreased iron stores. So far, results remain inconclusive [98], and little attention has been paid to the hypothesized intermediating role of iron in these studies. A huge drawback of many of these studies, however, is a type of bias called the healthy donor effect. This type of bias occurs when (healthy) donors are compared with non-donors. It is therefore recommended to conduct such health studies in blood donors within the active donor population [53], since comparisons within the donor population have shown to minimize healthy donor effects.

The current study is designed as a cross-sectional study within currently active whole-blood donors. We will investigate whether high-intensity (long duration, high frequency) blood donation is associated with reduced risk of MetS compared with low-intensity blood donation. Furthermore, we will explore if iron metabolism has a causal, intermediary role between blood donation and MetS. If iron metabolism is part of the causal pathway, over-adjustment bias should occur when we control for iron parameters in the analyses relating blood donation to MetS.

Materials and Methods

Study population

Between January 2011 and January 2012, each day a random sample of donors that would have been invited for a donation were instead invited to participate in the CARdiovascular risk and DONation (CARDON)-study. All donors were active whole-blood donors (i.e. at least one donation attempt in the last 2 years). This resulted in a random sample of 825 currently active whole-blood donors. A total of 781 donors (94.7%) responded to the invitation, of which 663 (80.4%) were willing to participate and 633 (76.7%) donors actually participated in the CARDON study. All participants provided written informed consent. The accredited Medical Research Ethics Committee Region Arnhem-Nijmegen approved the study's protocol, which is in accordance to the Declaration of Helsinki on ethical principles for medical research involving human subjects.

Measurements

Visits were scheduled between 8:00 and 10:30 am. All participants were instructed to abstain from smoking and to fast at least 8 hours before the visit. Information on lifestyle factors, familial history of cardiovascular disease and female reproductive factors were obtained by questionnaire. Anthropometric measurements were conducted in duplo according to protocols. Waist circumference was measured at the level midway between the lowest rib and iliac crest. The hip circumference was measured at the level of the head of the femur. Measurements started and ended with blood pressure measured manually (OMRON Digital

Blood Pressure Monitor, HEM-907 Intellisense, and WelchAllyn, Maxi Stabil 3) at the right arm in sitting position. Shoes, jackets/coats and heavy clothing were taken off during all measurements. Circumferences were adjusted by minus 2π times thickness of clothing. BMI (kg/m^2) was calculated by dividing weight in kilograms by squared height in metres. Donors brought the packaging of their medication used within 2 weeks before the visit with them. For analysing purposes, medication use was recoded according to the Anatomical Therapeutic Chemical classification system 2013 as provided by the World Health Organization [99].

Finally, fasting venous blood samples were drawn. Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, Apo-A1, Apo-B, ferritin, transferrin, iron, and high-sensitivity C-reactive protein (hs-CRP) were measured on an Olympus AU 400 (Beckman Coulter, Woerden, The Netherlands). Hb and mean corpuscular volume (MCV) were determined on the SysmexXT1800i (Sysmex Corporation, Kobe, Japan). Zinc protoporphyrine (ZPP) was measured on a ZPP Hematofluorometer (Aviv Biomedical, Inc., Lakewood NJ USA). Glucose and alanine aminotransferase (ALT) were both measured in plasma using Abbott reagents on the ARCHITECT C16000 (Abbott BV Diagnostics Division, Hoofddorp, The Netherlands). Insulin was determined in plasma with a two-step electrochemiluminescence immunoassay using a test kit from Roche Diagnostics on the Modular E170 (Roche/Hitachi Modular Analytics E170, Basel, Switzerland). Hepcidin was measured in Li-heparin plasma with an in-house competitive ELISA as described previously [75]. Some subjects (N=47) had plasma hepcidin levels below the lower limit of detection (0.216nM), and their values were therefore drawn randomly from a uniform distribution with minimum 0 and maximum 0.216 nM.

Definition of metabolic syndrome

According to the joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention [36], metabolic syndrome was considered present if a donor had at least three of the following five traits:

1. Elevated waist circumference (≥ 102 cm in men, ≥ 88 cm in women)

2. Elevated triglycerides (≥ 1.7 mmol/L or on drug treatment for elevated triglycerides)

3. Reduced HDL-C (< 1.0 mmol/L in men, < 1.3 mmol/L in women or on drug treatment for reduced HDL-C)

4. Elevated blood pressure (≥ 130 mm Hg systolic blood pressure or ≥ 85 mm Hg diastolic blood pressure or on antihypertensive drug treatment in a patient with a history of hypertension)

5. Elevated fasting glucose (≥ 5.6 mmol/L or on drug treatment for elevated glucose)

Table 2.2: Categorization of donation intensity into low, medium, or high based on sex-specific tertiles of donation career and donation frequency per year

	Donation frequency per year			
Men	First tertile ≤ 2.2	Second tertile 2.3 - 3.1	Third tertile > 3.1	
Donor career in years				Total
First tertile ≤ 16.2	Low N=50	Low N=28	Medium N=64	N=142
Second tertile 16.3 - 25.0	Low N=52	Medium N=56	High N=31	N=139
Third tertile > 25.0	Medium N=39	High N=56	High N=46	N=141
Total	N=141	N=140	N=141	N=422

	Donation frequency per year			
Women	First tertile ≤ 1.5	Second tertile 1.6 - 2.0	Third tertile > 2.0	
Donor career in years				Total
First tertile ≤ 12.0	Low N=19	Low N=19	Medium N=33	N=71
Second tertile 12.1 - 19.9	Low N=30	Medium N=21	High N=19	N=70
Third tertile > 19.9	Medium N=22	High N=30	High N=18	N=70
Total	N=71	N=70	N=70	N=211

Donation intensity

There is no consensus about the best way to measure donation exposure when comparing donors with different donation intensities. We have created an exposure measurement that combines the average frequency per year and the duration of the donor career. Duration of donor career was defined as the time between first and last donation, plus time until next allowed donation. Donation frequency per year was calculated by number of lifetime donations/duration of donor career. As men are allowed to donate more often than women (5 vs. 3 times a year), sex-specific tertiles were created for both variables. Donation intensity was subsequently created as three different levels, based on the sex-specific tertiles of donation frequency and career: low, medium, and high [Table 2.2]. Of note, a donation was counted if more than 100 ml of whole-blood was collected. In the Netherlands, donations are allowed at minimum intervals of 56 days. Also, donors must meet the criteria of $\geq 50 \leq 100$ mmHg diastolic and $\geq 90 \leq 180$ mmHg systolic blood pressure, and a capillary Hb level between $\geq 7.8 \leq 11.0$ mmol/l for women and $\geq 8.4 \leq 12.0$ mmol/l for men before donation. Sometimes whole-blood donors (temporarily) switch to plasmapheresis. Although our donors were all whole-blood donors at the time of inclusion, some had a history of plasmapheresis. When at least 30 ml was drawn during plasmapheresis, that donation was counted as a valid plasma donation. All donors with at least one valid plasma donation were categorized as 'ever having been a plasma donor'.

Statistical analysis

As our data are derived from a cross-sectional sample, the prevalence ratio is the most appropriate effect estimate to express a relative risk [100]. To allow for multivariate adjustment, robust Poisson regression (a.k.a. Poisson regression with a sandwich estimator of the variance) was used to estimate the association between donation intensity groups and metabolic syndrome [100]. This resulted in prevalence ratios with 95% confidence intervals (CIs). The lowest donation intensity was consistently used as the reference level of exposure. Unadjusted and adjusted models were built. We considered the following factors as potential confounders: age, smoking behaviour (status and pack years), body mass index (BMI), having ever been a plasma donor, hs-CRP, having a family history of cardiovascular disease and menopausal status.

Because of the dependency of BMI with waist circumference, one of the traits of the metabolic syndrome, ordinary conditional regression would over-adjust the intended effect estimate. Furthermore, BMI could also be a potential effect modifier. One could imagine that blood donation is primarily protective of MetS in obese subjects as opposed to non-obese subjects, because increased body fatness alters iron metabolism through adipocyte hormones [21]. Therefore, we have also performed our analyses within non-obese (BMI < 30 kg/m^2) and obese (BMI ≥ 30kg/m^2) donors separately. To examine the effect of iron metabolism, all iron parameters, the ratios of hepcidin to ferritin and of hepcidin to TS, were included as continuous variables one-by-one in the adjusted model. Also, the simultaneous

inclusion of hepcidin and ferritin into the adjusted model was examined. All analyses were conducted with IBM SPSS Statistics 21, Release Version 21.0.0.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Mac, Version 21.0. Armonk, NY, USA: IBM Corp.).

Results

Of 633 participating donors, 422 (66.7%) were men and 211 were women (33.3%). Across the donation intensity groups, donors differed in age, smoking status, BMI, having ever been a plasma donor, hs-CRP, having a family history of cardiovascular disease, menopausal status, and lifetime number of Hb and blood pressure deferrals [Table 2.3]. In men, a dose-response relation between donation intensity and iron depletion was present, whereas such an effect was less pronounced in women [Table 2.3]. A total of 145 donors (22.9%) were classified as cases of having Metabolic Syndrome (MetS). Prevalence of MetS surprisingly increased with increasing donation intensity in both sexes [Table 2.4].

Within men, there was no association between donation intensity and MetS, with an adjusted prevalence ratio for medium- and high-intensity donors of 1.09 (95%-CI: 0.69 to 1.73) and 1.46 (95% CI: 0.93 to 2.30), respectively [Table 2.4].

In female donors, prevalence ratios were higher than in men. Prevalence ratios attenuated to 1.99 (95%-CI: 0.87 to 4.52) and 2.14 (95%-CI: 0.94 to 4.86) for donors with medium and high donation intensity upon controlling for confounding factors, respectively.

The analyses within non-obese and obese subjects revealed that the positive association between donation intensity and MetS was mainly restricted to non-obese female donors, and that iron parameters were not involved [Supporting Information -Table A1].

Although these results point towards a higher risk of MetS in high-intensity donors, we did perform the proposed analyses that would investigate whether iron metabolism has any part in these associations. The iron parameters with the strongest influence (MCV, ferritin, and the simultaneous inclusion of ferritin and hepcidin) on the adjusted prevalence ratios are presented in Table 2.4. These analyses revealed that the iron parameters barely affected the adjusted prevalence ratios in either men or women [Table 2.4].

Next, we let go of our donation intensity grouping, and explored the two main iron metabolism parameters ferritin and hepcidin, and how they related to MetS prevalence. Separately for men and women, ferritin and hepcidin medians were used to create four different groups: low ferritin and low hepcidin (1); high ferritin and low hepcidin (2); low ferritin and high hepcidin (3); high ferritin and high hepcidin (4). Within men, increased prevalence of MetS seems predominantly affected by high ferritin, whereas in women high hepcidin appeared to be associated with increased prevalence of MetS [Figure 2.3].

Table 2.3: Characteristics of donors across donation intensity groups

Characteristic	Donation intensity Low N=198	 Medium N=235	 High N=200
Donor career (y)	12.6 (6.8)	15.3 (9.7)	25.5 (4.3)
Donation frequency/year	1.6 (0.6)	2.6 (1.1)	2.7 (0.6)
Ever been plasma donor	17 (8.6%)	16 (6.8%)	23 (11.5%)
Lifetime deferrals for			
Low Hb	100 deferrals	115 deferrals	148 deferrals
0 deferrals	150 (75.8%)	176 (74.9%)	138 (69.0%)
1 deferral	22 (11.1%)	33 (14.0%)	24 (12.0%)
2 deferrals	12 (6.1%)	11 (4.7%)	17 (8.5%)
≥ 3 deferrals	14 (7.1%)	15 (6.4%)	21 (10.5%)
Blood pressure	13 deferrals	19 deferrals	24 deferrals
0 deferrals	190 (96.0%)	220 (93.6%)	184 (92.0%)
1 deferral	5 (2.5%)	12 (5.1%)	11 (5.5%)
2 deferrals	1 (0.5%)	2 (0.9%)	3 (1.5%)
≥ 3 deferrals	2 (1.0%)	1 (0.4%)	2 (1.0%)
Age (y)	54.2 (5.7)	54.2 (5.6)	57.1 (5.8)
BMI (kg/m^2)	26.1 (3.1)	26.7 (3.8)	27.1 (4.0)
Underweight (< 18.5)	0 (0%)	0 (%)	1 (0.5%)
Normal (18.5 - 24.99)	80 (40.4%)	87 (37.0%)	57 (28.5%)
Overweight (≥ 25)	98 (49.5%)	107 (45.5%)	107 (53.5%)
Obese (≥ 30)	20 (10.1%)	41 (17.4%)	35 (17.5%)
Postmenopausal status	42 (61.8%)	45 (59.2%)	50 (75.8%)
Smoking status			
Never	61 (31.0%)	75 (32.1%)	71 (35.9%)
Ever	102 (51.8%)	116 (49.6%)	100 (50.5%)
Current	34 (17.3%)	43 (18.4%)	27 (13.6%)
Pack years[a] (y)	10.0 (11.6)	9.7 (14.9)	10.0 (12.5)
Blood pressure (mmHg)			
Systolic	128 (15)	131 (16)	136 (17)
Diastolic	82 (9)	84 (9)	86 (8)
Waist (cm)	94.3 (9.6)	95.7 (10.8)	97.4 (12.2)
Waist-to-hip ratio	0.94 (0.07)	0.93 (0.08)	0.95 (0.08)

[a] Median (interquartile range). [b] HOMA-IR, Homeostasis model assessment-insulin resistance, calculated as $(insulin * glucose)/22.5$. Hb, haemoglobin; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC/HDL ratio, total cholesterol/HDL ratio; Apo A1, apolipoprotein A-I; ApoB, apolipoprotein B; ALT, alanine aminotransferase; hs-CRP, high-sensitivity c-reactive protein; CVD, cardiovascular disease; AMI, acute myocardial infarction. Transferrin saturation is calculated by $(100 * total\ iron)/(transferrin * 25)$.

Table 2.3: Characteristics of donors across donation intensity groups (continued)

Characteristic	Donation intensity		
	Low	Medium	High
	N=198	N=235	N=200
Triglycerides (mmol/l)	1.39 (1.37)	1.43 (0.80)	1.37 (0.71)
Cholesterol (mmol/l)			
Total cholesterol	5.56 (1.03)	5.64 (1.08)	5.55 (0.95)
HDL-cholesterol	1.43 (0.33)	1.38 (0.33)	1.41 (0.34)
LDL-cholesterol	4.10 (0.84)	4.16 (0.91)	4.13 (0.82)
TC/HDL ratio	4.07 (1.23)	4.28 (1.16)	4.10 (1.00)
Apo A-I (g/l)	1.56 (0.21)	1.52 (0.21)	1.54 (0.22)
ApoB (g/l)	0.99 (0.21)	1.01 (0.21)	0.99 (0.18)
Glucose (mmol/l)	5.2 (0.7)	5.2 (0.6)	5.3 (0.6)
HOMA-IR[b] (mmol/l)	2.2 (1.8)	2.3 (1.6)	2.6 (2.0)
ALT[a] (U/l)	24 (13)	26 (13)	25 (13)
hs-CRP[a] (mg/l)	1.03 (1.76)	1.10 (1.82)	1.21 (1.71)
Lipid lowering meds	11 (5.6%)	12 (5.1%)	14 (7.0%)
Anti-hypertensive meds	30 (15.2%)	38 (16.2%)	39 (19.5%)
Glucose lowering meds	5 (2.5%)	4 (1.7%)	3 (1.5%)
Familiy history of CVD	101 (51.0%)	134 (57.0%)	110 (55.0%)
AMI	69 (34.8%)	95 (40.4%)	74 (37.0%)
Stroke	48 (24.2%)	69 (29.4%)	57 (28.5%)
Haemoglobin (mmol/l)			
Men	9.4 (0.5)	9.4 (0.5)	9.4 (0.6)
Women	8.6 (0.5)	8.6 (0.5)	8.7 (0.5)
Transferrin saturation (%)			
Men	30 (14)	27 (12)	26 (12)
Women	28 (16)	26 (11)	28 (12)
Ferritin[a] (μg/l)			
Men	48.3 (42.0)	35.7 (30.1)	35.7 (29.2)
Women	39.5 (34.7)	36.1 (34.3)	36.4 (33.6)
Hepcidin[a] (nM)			
Men	2.24 (2.92)	1.60 (2.68)	1.14 (1.77)
Women	1.74 (2.62)	1.91 (2.38)	1.98 (2.69)

[a] Median (interquartile range). [b] HOMA-IR, Homeostasis model assessment-insulin resistance, calculated as (insulin * glucose)/22.5. Hb, haemoglobin; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC/HDL ratio, total cholesterol/HDL ratio; Apo A1, apolipoprotein A-I; ApoB, apolipoprotein B; ALT, alanine aminotransferase; hs-CRP, high-sensitivity c-reactive protein; CVD, cardiovascular disease; AMI, acute myocardial infarction. Transferrin saturation is calculated by $(100x totaliron)/(transferrin x 25)$.

Table 2.4: Prevalences and Prevalence Ratios (95%-CIs) of Metabolic Syndrome

Sex	Donation Intensity	MetS N(%)	Prevalence Ratio (95%-CI)				
			Unadjusted	Adjusted[a]			
					+MCV	+Ferritin	+Ferritin +Hepcidin
Men	Low N=130	23 (20.0)	Ref.	Ref.	Ref.	Ref.	Ref.
	Medium N=159	34 (21.4)	1.07 (0.68-1.69)	1.09 (0.69-1.73)	1.09[b] (0.69-1.74)	1.20[b] (0.75-1.92)	1.18 (0.74-1.90)
	High N=133	40 (30.1)	1.50 (0.98-2.31)	1.46 (0.93-2.30)	1.47[b] (0.93-2.32)	1.71[b] (1.07-2.74)	1.70 (1.06-2.72)
Women	Low N=68	7 (10.3)	Ref.	Ref.	Ref.	Ref.	Ref.
	Medium N=76	19 (25.0)	2.43[b] (1.09-5.42)	1.99 (0.87-4.52)	1.79 (0.79-4.08)	2.07 (0.92-4.66)	2.06 (0.91-4.71)
	High N=67	19 (28.4)	2.76[b] (1.24-6.12)	2.14 (0.94-4.86)	1.99 (0.88-4.52)	2.33 (1.04-5.26)	2.29 (1.01-5.20)

[a] Adjusted model includes: age (continuous), hs-CRP (continuous), smoking (categorical, 3 levels), having ever been a plasma donor (dichotomous), having a family history of CVD (dichotomous), and postmenopausal status (dichotomous). [b] Model did not converge. MCV, mean corpuscular volume; hs-CRP, high-sensitivity C-reactive protein; CVD, cardiovascular disease.

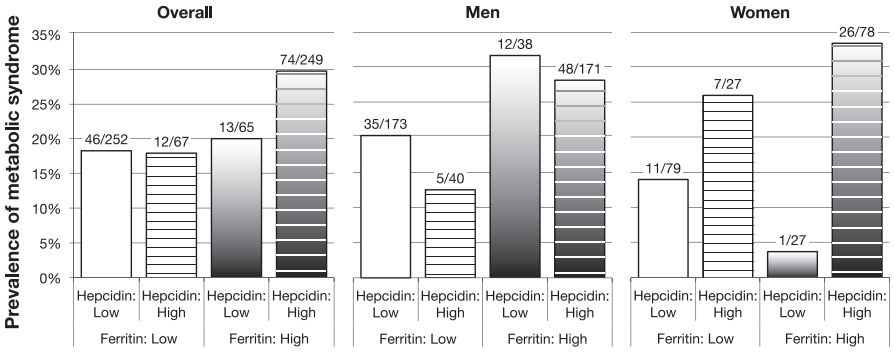

Figure 2.3: Prevalence (n/N) of metabolic syndrome according to strata based on sex-specific medians of ferritin (men: 38.13, women: 37.09 μg/l) and hepcidin (men: 1.62, women: 1.91 nM).

Discussion

This study indicates an association between high-intensity blood donation and metabolic syndrome (MetS), independent of iron metabolism. In both sexes, prevalence of MetS was approximately 1.5 to 2.5 times higher in high intensity donors compared with low intensity donors. Of note, after adjusting for confounders, no significant effect remained. Moreover, the remaining twofold increase in MetS prevalence in women could not be further explained by any iron parameter. These findings are contrary to what would be expected: donors with a high frequency of donations per year over a long period of time should have prolonged iron depletion, which would prevent them from developing MetS.

Although there are many studies that have found a positive association between increased iron stores and MetS, the causal sequence is currently being debated [33, 34]. The potential role of obesity has had little attention in previous studies, although it is highly likely to be associated with MetS and probably also with blood donation intensity. This is possibly due to the pre-donation criterion for body weight of at least 50 kg, hindering lean donors from donating more often. Furthermore, heavy weight donors could probably carry the burden of a donation more easily, as the drawn volume is the same for all whole-blood donors. The association between obesity and MetS is easily derived from the inclusion of waist circumference as a trait of MetS; both obesity and waist circumference are strongly related to central adiposity. Adipose tissue and two of its secretory hormones, adiponectin and leptin, have recently been proposed to play a role in iron metabolism and insulin resistance. Adiponectin is decreased with increasing body fatness and is inversely correlated with ferritin and transferrin as well as adipose tissue insulin resistance [39, 94]. Leptin level is directly proportional to the amount of body fat, and was found to up-regulate hepcidin transcription in hepatocytes [93]. Furthermore, in a recent study including three independent cohorts, iron seems to accumulate in adipose tissue with increasing body fatness and impaired insulin action [35].

These novel findings indicate that obesity could be associated with increased (adipocyte) iron stores and insulin resistance, and this has made us aware of the potential effect-modifying properties of obesity in the relation between blood donation and MetS. Meaning that obese donors could especially be susceptible to the benefits of lowering iron stores through blood donation, as they would have increased (adipocyte) iron retention. In contrast, one would expect to find a more reliable and "true" effect of blood donation in non-obese donors who have a normal, regular iron metabolism. We have found that the increased prevalence of MetS in high-intensity blood donors was mainly restricted to non-obese female donors, indicating that obesity is indeed an effect modifier of blood donation and MetS prevalence, but not in the way that we anticipated. In obese donors, there was no association between blood donation and MetS prevalence. In either group, iron parameters did not explain the (lack of) association between blood donation and MetS. These results do not support the novel interplay between iron and adipose tissue in the same direction.

There is no doubt that iron, adipose tissue, and insulin resistance keep each other in a vicious cycle [21], making it difficult to assess direct causal effects in any study. On top of that, our study has a cross-sectional design, that does not allow us to study the temporal relationship between blood donation, iron, and MetS, although it is at least highly likely that iron levels have decreased due to frequent blood donation. We propose that in epidemiological studies, MetS is not just an intermediary factor of the relation between blood donation (and thus lower iron stores), and cardiovascular disease, but is also an important confounding factor. Meaning that lower MetS risk is not just an effect of lower iron stores through blood donation, but that MetS (or some of its components) also affects (the probability of) blood donation. Moreover, alternative causal pathways of blood donation leading to a decreased cardiovascular risk, such as a reduced viscosity, have not been addressed in this study [41].

Previous studies showing a protective effect of blood donation on cardiovascular disease were often concerned about a healthy donor effect bias when comparing donors with the general population or ex-donors. To minimize the healthy donor effect, we have embedded our study within currently active whole-blood donors as recommended [53]. Furthermore, our main finding (increasing MetS prevalence in high-intensity donors) supports the absence of a healthy donor effect. The fact that both medium- and high-intensity female non-obese donors had 3.5-fold increased prevalence of MetS does, however, leave the possibility of residual confounding. In a post-hoc analysis, we did not find differences in physical activity or meat consumption across donation intensity groups.

Another strength of our study is the inclusion of women in addition to the inclusion of men. This seems to become even more important now that gender differences are surfacing in iron metabolism and insulin resistance and MetS [101, 102]. Hepcidin is the key regulatory peptide of iron metabolism and could be of particular importance in the development of MetS as it also regulates the iron content of macrophages and liver cells [31, 103]. Increased cellular iron could decrease hepatic insulin extraction and impair insulin signalling [34, 62], leading to hyperinsulinemia. Furthermore, iron-catalysed formation of reactive oxygen species could damage pancreatic β-cells, inducing insulin resistance [34, 58]. Cellular iron is entrapped within cells by higher levels of hepcidin [23]. With frequent blood donation, hepcidin is being down-regulated in response to lower iron stores and increased erythropoiesis, releasing cellular iron from erythrophagocytosed macrophages which can thus become available for erythroid precursors [103].

Hepcidin levels could therefore in particular be involved in the relation between blood donation and MetS, which would be in agreement with our observation in women. The apparent importance of hepcidin in women with MetS was also found in a population-based study conducted by Martinelli et al., in which MetS was an independent predictor of hepcidin, even after adjustment for ferritin [101]. Interestingly, we found ferritin to be the main explanatory factor of MetS in men, a phenomenon that has also been observed by Kim et al. in normal fasting glucose men but not in women [104]. In a 5-year follow-up study, higher ferritin levels were also associated with future MetS in a Korean population of healthy

men at baseline [63]. In contrast, Sheu et al. found that ferritin was associated with insulin resistance in Chinese non-diabetic women but not in non-diabetic men [102]. Thus, the mechanism that underlies the gender difference is poorly understood yet.

To ensure sufficient iron depletion, this study was conducted within currently active donors. This is confirmed by quite low iron stores, with hepcidin ranges even lower than those observed in a population-based study [68] and in a comparable study of whole-blood donors [105]. As MetS is a reversible condition, maybe a more recent level of exposure is more important. Therefore, we have also used the number of donations in the last 2 years before study participation categorized into sex-specific tertiles in a post-hoc analysis. These donation tertiles were not consistently associated with MetS prevalence, and iron parameters were accordingly marginally able to explain the observed differences in MetS across donation tertiles. Thus, it could be that the effects of iron depletion are more short-term than we expected. On the other hand, phlebotomy has been repeatedly opted as a safe and cost-effective treatment for patients with dysmetabolic iron overload syndrome, and also for NAFLD [33, 34], and our chosen exposure measurement of donation intensity should reflect the potential benefit of such a treatment in a healthy population. A randomized trial in MetS patients has already shown a positive effect on blood pressure after phlebotomy-induced reduction of body iron stores, but not on insulin resistance [97].

In conclusion, this study showed that high-intensity blood donation is not associated with a decreased risk of MetS. Within non-obese female donors, donation intensity was positively associated with MetS prevalence, independent of iron parameters. However, increased prevalence of MetS was mainly associated with higher ferritin in men, whereas high hepcidin predominantly affected MetS prevalence in women. Future research should address these gender differences and the role of obesity herein, and longitudinal data is needed to better value the potential of blood donation in preventing MetS.

Acknowledgments

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Competing interests

The authors have no competing interests.

Author contributions

K.P. collected, interpreted, and analyzed the data, wrote, reviewed, and edited the manuscript. F.A. co-ordinated the study, contributed to study design, interpreted the data, contributed to discussion, and reviewed the manuscript, A.L.M.V. and M.H. contributed to study design, interpreted the data, contributed to discussion, and reviewed the manuscript, D.W.S. contributed to study design and reviewed the manuscript, A.J.G.-M. provided assistance with data collection. K.P. and F.A. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

Chapter 3

Subclinical Atherosclerosis

Blood donors are not allowed to have overt cardiovascular disease. Yet, in our quest of the preventive ability of blood donation on cardiovascular disease, it is very important to know whether early, subclinical deteriorations are making its presence in the less-active blood donor. Nowadays, non-invasive measurements of atherosclerosis exist that are able to shed some light on the presence of these subclinical deteriorations. These measurements reflect resistance, stiffness, and thickening of the vascular wall, anatomical deviations of which that are all involved in the pathology of atherosclerosis. Will low-frequency blood donors already have an impaired vascular integrity than high-frequency blood donors?

3.1 Vascular Integrity

Original manuscript title:
The effect of frequent blood donation on ferritin, hepcidin, and subclinical atherosclerosis.
K. Peffer, M. den Heijer, S. Holewijn, J. de Graaf, D. W. Swinkels, A. L. M. Verbeek, F. Atsma
Transfusion 2013, 53(7), pp. 1468-1474.

BACKGROUND: Iron catalyzes the formation of free radicals, which could lead to damaged vascular walls and subsequent atherosclerosis. Blood donation decreases iron stores and can thus decrease cardiovascular risk. Even within blood donors, differences in stored iron are observed. This study investigates whether increasing lifetime number of donations decreases the extent of subclinical atherosclerosis within blood donors.
STUDY DESIGN AND METHODS: Subclinical atherosclerosis was evaluated in 269 blood donors by measuring intima-media thickness (IMT), pulse-wave velocity (PWV), and ankle-brachial index (ABI). Lifetime number of whole-whole-bloodblood donations was categorized into sex-specific donation tertiles.
RESULTS: Ferritin and hepcidin were lower in high- frequency donors compared to low-frequency donors. Donors in the third sex-specific donation tertile had on average a 0.3% (95% confidence interval [CI], -3.6 to +3.0%) lower IMT, a 2.1% (95% CI, -3.9 to +8.0%) higher PWV, and a 1.5% (95% CI, -1.4 to +4.5%) higher ABI compared to donors in the first sex-specific donation tertile.
CONCLUSION: With such small differences and no consistent trend across donation groups, it cannot be concluded that blood donation has a beneficial effect on the extent of subclinical atherosclerosis.

Introduction

In 1981, Sullivan [8] proposed protective effects of depleting iron stores on heart disease. He arrived at the iron hypothesis by three main observations: 1) myocardial failure in iron storage diseases, 2) accumulation of stored iron with age in men, and 3) accumu- lation of stored iron in postmenopausal women to levels found in men. With these findings he aimed to explain the established difference in cardiovascular risk between men and women. Due to the cessation of menstrual blood loss with menopause, and hence increasing iron stores, post-menopausal women would rapidly deteriorate in cardiovascular risk and approach that observed in men.

Iron has the potential to catalyze the formation of reactive oxygen species, of which hydroxyl radicals are of major concern [16]. Hydroxyl radicals oxidize low-density lipoprotein (LDL) cholesterol, which leads to the formation of foam cells and ultimately atherosclerosis [4]. If no such reactive iron is available, as in iron depletion, atherosclerosis may be prevented or its progression slowed. Over the past years, more and more insight has been gained into iron metabolism, of which the discovery of hepcidin has been of major importance. This peptide hormone appears to play a key role in iron homeostasis and disorders [60] and is therefore of additional value when assessing iron status.

Iron depletion refers to a state of decreased storage iron and hemoglobin (Hb) within the normal range. This condition is likely to be more common in whole-blood donors than in the general population. Therefore, blood donors comprise an effective study population to investigate the relationship between depleted iron stores and cardiovascular risk. Particularly high-frequency blood donors have been shown to have decreased body iron levels compared to low-frequency donors [1, 2, 69, 106]. However, previous epidemiologic studies on associations between iron depletion and cardiovascular risk remain inconclusive [45–47, 107]. Although the epidemiologic studies reported by Kiechl and coworkers [107], Meyers and coworkers [47], and Salonen and coworkers [45] concluded that lowered body iron was protective against cardiovascular disease, Ascherio and colleagues [46] concluded otherwise.

All of these studies mainly used cardiovascular events and/or deaths as outcome measurements, whereas others used endothelial dysfunction [49, 51], which is related to progression of atherosclerosis [108–110]. One of these two studies using endothelial dysfunction as outcome measurement found that frequent blood donors have an improved vascular function compared to occasional blood donors [49], as measured by flow-mediated dilation and intima-media thickness (IMT), whereas the other one only found a small nonsignificant difference [51]. However, drawbacks of these studies are a small sample size [49], self-reported donation history [51], and no sex-specific cutoff for number of donations [49, 51]. The latter factor is important as men are allowed to donate five times a year whereas women are restricted to a maximum of three donations per year.

To obtain valid estimates of association, the current study is designed as a population-based cohort of whole-blood donors with donation data obtained from the blood bank. Using sex-specific cutoffs for lifetime number of donations, we compared high-frequency donors with low-frequency donors regarding the extent of subclinical atherosclerosis.

Materials and Methods

Study population

Data were obtained from the Nijmegen Biomedical Study (NBS), a population-based survey conducted by the Department of Epidemiology and Biostatistics and the Department of Clinical Chemistry of the Radboud University Nijmegen Medical Centre. In accordance with the Declaration of Helsinki, the study protocol was approved by the institutional review board of the Radboud University Nijmegen Medical Centre. All participants provided written informed consent [111].

The first part of the NBS, NBS-1, included an age- and sex-stratified random sample of 22,451 adults selected from the Population Registry of Nijmegen. Between August 2002 and December 2003, a total of 21,756 subjects were sent a postal questionnaire, of whom 9350 (43%) responded and provided written informed consent. Part 2 of the NBS (NBS-2) was conducted 3 years after the commencement of NBS-1 by the Department of Internal Medicine. In NBS-2, all 2114 middle-aged subjects (50-70 years) from the NBS-1 cohort were reinvited. From this group, 1491 subjects participated in NBS-2 (response 71%).

These NBS-2 participants were linked to Sanquin's blood bank registry based on sex, date of birth, and last name. In case of multiple matching, residency was used additionally in the linkage strategy. Of the 1491 NBS-2 participants, 272 persons could be linked to the blood bank registry. Three of them were excluded: one individual started donating blood after participating in NBS-2 and one individual appeared to be a bone donor. A third participant was excluded because of lipid-lowering medication use within the preceding 4 weeks of the measurements. This left 269 donors available for data analysis (152 men, 117 women).

Data collection

The following data from NBS-2 were used in the present study: non-invasive measurements of atherosclerosis (NIMA), blood variables, and a self-administered questionnaire. NIMA measurements consisted of carotid IMT of the common carotid artery, ankle-brachial index (ABI), and pulse-wave velocity (PWV). All measurements were taken with participants in supine position after at least 10 minutes' rest in a temperature-controlled room (23-24°C) and performed by well-trained and certified sonographers according to highly standardized protocols as previously described [112].

IMT was measured with an ultrasound machine (AU5, Esaote Biomedica, Genova, Italy) with a 7.5-MHz linear-array transducer. Actual measurement of the IMT was performed off-line by the sonographer at the time of the examination, using semiautomatic edge-detection software (M'Ath, Standard Version 2.0, Metris, Argenteuil, France). Averaging the mean of all four measured segments (far wall left, near wall left, far wall right, and near wall right) of the distal common carotid artery yielded the mean IMT in millimeters.

PWV was determined by applanation tonometry, using a commercially available noninvasive central blood pressure assessment system (SphygmoCor, Version 7.1, Atcor Medical, Sydney, Australia). Pulse waveforms were recorded at two sites sequentially (right carotid

artery and left femoral artery), and wave transit time was calculated using the R-wave of a simultaneously recorded electrocardiogram as a reference frame as described before [113].

For ABI, appropriately sized cuffs were placed around both arms above the elbow and around both legs just above the ankle. Resting blood pressures were mea- sured at the left and right brachial artery and the left and right posterior tibial and dorsalis pedis arteries using an 8-MHz hand-held Doppler probe (ImexDop CT+, Biomedic, Almere, The Netherlands). The lowest of the four ABIs as calculated by dividing the four ankle pressures by the highest of the two arm pressures was used in the analysis.

Blood samples were collected from all participants in both NBS-1 and NBS-2 and drawn after an overnight fasting or in the afternoon 6 hours after a standardized breakfast of 400 kcal (where 1 kcal = 4.184 kJ). Breakfast was standardized by instructing participants to consume a maximum of two slices of brown bread with jam or honey and a glass of milk at least 6 hours before the visit. All participants were specifically instructed not to smoke or use tea, coffee, chocolate, or alcohol for at least 12 hours before the visit and asked to discontinue any lipid-lowering medication 4 weeks before measurements. Adherence to these instructions was assessed by thoroughly questioning the participants; those who admitted to have violated the instructions were excluded from analysis.

Total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (TG), and glucose were determined in blood samples. LDL cholesterol was calculated according to the Friedewald method [114]. Since no iron variables were measured in NBS-2, levels of hepcidin, ferritin, transferrin saturation, iron, and iron-binding capacity as well as alanine aminotransferase (ALT), and C-reactive protein were retrieved from NBS-1. Hepcidin was measured by competitive enzyme-linked immunosorbent assay as described in Galesloot and colleagues [68] and Kroot and colleagues [75].

Furthermore, height and weight were measured to obtain the body mass index (BMI), calculated as body weight (in kg) divided by squared height (in m). Systolic blood pressure (SBP) and diastolic blood pressure were measured using an oscillometric sphygmomanometer (Criticon model no. 1846, Criticon, Inc., Tampa, FL). The self-administered questionnaire provided data on cardiovascular disease history, medication use, and smoking behavior. Prevalent cardiovascular disease was defined as a reported myocardial infarction, transient ischemic attack, stroke, peripheral arterial disease, coronary artery bypass or angioplasty, or treated angina. Diabetes mellitus 2 (DM2) was defined as fasting glucose of at least 7 mmol/L or when previously diagnosed and treated DM2. Presence of diabetes mellitus 1 (DM1) was based on self-reported data. Smoking behaviour was classified as current, ever, or never.

Donation data

As the donor population consists of donors providing both plasma and whole-blood, we only considered whole-blood donations to contribute to the amount of iron depletion. As a result, the lifetime number of donations represents the total amount of whole-blood donations per donor. A whole-blood donation was counted as valid when the drawn volume was at least 100 mL. Before 2000, data on type of donation (e.g., whole-blood or plasma) and drawn blood volume are limited. To estimate the donation type of those unknown, we assumed that an interval of at least 56 days between the two donation dates would imply a whole-blood donation. When this interval was shorter, a different type of donation was assumed (e.g., plasma donation or a deferral) and was therefore not counted. Missing drawn volumes were assumed as being sufficient ($\geq$ 100 mL) and accompanying donations were treated as effectual donations. All valid donations until subclinical atherosclerosis measurements were performed were added up. The resulting lifetime number of donations was categorized into sex-specific tertiles (tertiles in men: $\leq$ 28 donations, 29-47 donations, $>$ 47 donations; tertiles in women: $\leq$ 15 donations, 16-27 donations, $>$ 27 donations).

Statistical analysis

First, descriptive statistics were performed. Differences in baseline characteristics between donors in the three sex- specific donation tertiles were assessed with chi-square tests, analysis of variance, and the Kruskal-Wallis test.

Second, linear regression models were used to analyze the relation between sex-specific donation tertiles and each NIMA outcome (IMT, PWV, and ABI). Mean differences in NIMA variables between the three sex-specific tertiles were calculated with corresponding 95% confidence intervals (95% CIs). Three different models were built: Model 0 calculated unadjusted differences, in Model 1 differences were adjusted for sex and age, and in Model 2 differences were additionally adjusted for smoking status, BMI, SBP, DM1 and DM2, total cholesterol, HDL, and TG. All analyses were performed using computer software (PASW Statistics 19, Release Version 19.0.0, SPSS, Inc., 2010, Chicago, IL, http://www.spss.com).

Results

Table 3.1 shows the characteristics of the study population per sex-specific donation tertile (n=269). Of 269 blood donors, 152 (57%) were men and 117 (43%) were women. Mean (±SD) age was 61.2 (±5.6) years for men and 59.8 (±5.6) years for women. High- and low-frequency donors mainly differed in smoking behavior and the time elapsed between their last whole-blood donation and participating in NBS-2. Ferritin was lower with increasing lifetime number of donations, but the proportion of iron depleted subjects did not differ between donors in the first and second sex-specific donation tertile. Within men, 6.8% were iron depleted within the third donation tertile, whereas the proportion of female donors with iron depletion was 20.0%.

Hepcidin was also inversely related to number of donations, with the lowest median observed in male donors within the third donation tertile (4.6 nmol/L).

Regression analysis resulted in adjusted mean differences expressed as percentage change from the first sex-specific donation tertile [Table 3.2]. The small differences found between the three donation groups in Model 0 and Model 1 reduced to the null when fully adjusted in Model 2. Mean IMT was only -0.3% (95% CI, -3.6 to +3.0%) lower in donors in the third sex-specific donation tertile compared to those in the first sex-specific donation tertile. As for PWV, donors in the third sex-specific donation tertile had a slightly higher mean value of +2.1% (95% CI, -3.9 to +8.0%) than donors in the first sex-specific donation tertile. Mean ABI scores were just +1.5% (95% CI, -1.4 to +4.5%) higher in donors in the third sex-specific donation tertile compared to donors in the first sex-specific donation tertile. Overall, none of the NIMA variables showed a consistent and/or significant trend across the three donation groups.

Discussion

This study did not reveal a clear effect of lifetime number of whole-blood donations on the extent of subclinical atherosclerosis measured as IMT, PWV, and ABI as only very small differences were found across sex-specific donation tertiles without a consistent pattern of association. We confirmed that blood donation decreases iron stores, as increasing number of donations was strongly associated with lower ferritin and hepcidin distributions. Although our sex-specific donation tertiles seem to distinguish different levels of exposure, we did not find an effect of blood donation on the extent of subclinical atherosclerosis. As our measurements of iron status depend on a single determination, they cannot provide information about lifelong exposure levels. Repeated iron measurements throughout a donor's career would have been informative. Because our sex-specific donation tertiles were so closely correlated to iron levels, they might also serve as a proxy measurement of lifelong exposure status. By comparing the exposure window from first to last donation between our donation tertiles, it appears that donors in the third sex- specific donation tertile not only have a higher number of lifetime donations, but also a longer time period of exposure [Table 3.1]. This indicates that donors in the third sex-specific donation tertile are at a higher and longer exposure level than donors in the first and second sex-specific donation tertiles.

Several underlying mechanisms of iron affecting the development of cardiovascular disease have been proposed. Initially, research focused on storage iron being redox active, subsequently enabling LDL oxidation. Recently, Sullivan [31] proposed an additional mechanism in which hepcidin can promote progression of atherosclerotic plaque by slowing or preventing the mobilization of iron from macrophages within the atherosclerotic plaque. Hepcidin is a newly emerged key hormone in iron balance, which binds and subsequently degrades the cellular iron exporter ferroportin, leaving iron trapped inside the iron-containing cells. As a result of blood donation, erythropoiesis occurs, which in turn down regulates hepcidin expression, causing cellular iron export into plasma.

Table 3.1: Characteristics of donor population[a]

Characteristic	Lifetime number of donations[b]			p value
	First tertile (n=88)	Second tertile (n=89)	Third tertile (n=92)	
Age (y)	59.9 (±5.5)	60.8 (±5.7)	61.2 (±5.8)	0.321
Sex (men)	49 (55.7)	51 (57.3)	52 (56.5)	0.997
Time since last donation (y)	9.7 (±6.0)	6.0 (±4.1)	1.4 (±2.5)	0.000
Time between first and last donation (y)	7.3 (±5.4)	14.8 (±3.8)	20.8 (±3.7)	0.000
Ever deferred	23 (26.2)	38 (42.7)	46 (50)	0.004
Deferred for low Hb	8 (9.1)	8 (9.0)	10 (10.9)	0.890
One deferral for low Hb	8	7	5	
Two deferrals for low Hb	0	1	2	
Three deferrals for low Hb	0	0	3	
BMI (kg/m^2)	26.5 (±3.7)	27.4 (±4.7)	26.9 (±3.6)	0.332
Smoking status				0.027
Current	23 (26.2)	14 (15.7)	8 (8.7)	
Former	46 (52.3)	48 (53.9)	53 (57.6)	
Pack-years (y)	11.6 (±13.6)	10.3 (±14.2)	8.4 (±11.6)	0.256
Cholesterol (mmol/L)				
Total	5.86 (±0.94)	5.82 (±0.99)	5.81 (±1.01)	0.936
HDL	1.40 (±0.37)	1.36 (±0.42)	1.42 (±0.35)	0.608
TG (mmol/L)	1.36 (±0.71)	1.58 (±0.92)	1.35 (±0.67)	0.073
Blood pressure (mmHg)				
Systolic	126.7 (±13.9)	125.5 (±13.0)	128.8 (±14.1)	0.198
Diastolic	77.2 (±9.4)	77.6 (±9.6)	79.8 (±9.2)	0.155
Diabetes				0.065
No	81 (92.0)	80 (89.9)	91 (98.9)	
DM1	1 (1.1)	0 (0)	0 (0)	
DM2	6 (6.8)	9 (10.1)	1 (1.1)	
CRP (mg/L, n=78; 85; 84)	4.87 (±3.13)	4.64 (±1.90)	4.61 (±2.46)	0.768
ALT (U/L, n=78; 84; 84)	13.9 (±6.7)	15.1 (±7.6)	13.0 (±6.7)	0.168
Ferritin (μg/L)[c]				
Men (n=44; 50; 44)	116.9 (122.3)	78.2 (100.7)	33.0 (35.1)	0.000
Women (n=35; 34; 40)	53.5 (69.1)	60.8 (107.3)	32.4 (41.6)	0.000
Iron depleted (%)[d]				
Men	0	0	6.8	0.038
Women	2.9	2.9	20.0	0.012
Hepcidin (nmol/L)[c]				
Men (n=40; 43; 41)	18.2 (12.5)	11.8 (13.9)	4.7 (7.0)	0.000
Women (n=29; 26; 33)	17.7 (16.3)	10.4 (16.5)	8.7 (15.4)	0.025

Hb, haemoglobin; BMI, body mass index; HDL, high-density lipoprotein; TG, triglycerides; DM1/2, type 1/2 diabetes mellitus; CRP, C-reactive protein; ALT, alanine aminotransferase. [a] Data are reported as mean (±SD) or number (%). [b] Lifetime number of whole-blood donations divided into sex-specific tertiles. In men: ≤ 28 donations, 29-47 donations, > 47 donations; in women: ≤ 15 donations, 16-27 donations, > 27 donations. [c] Median (interquartile range). [d] Iron depletion defined as ferritin < 12 μg/L.

Table 3.2: Percentage difference (95% CI) between donation groups

	Model[a]		
Outcome	0	1	2
IMT (mm)	0	1	2
First tertile	Ref.	Ref.	Ref.
Second tertile	+0.2 (-3.4 to +3.8)	-0.5 (-3.8 to +2.9)	+0.2 (-3.1 to +3.5)
Third tertile	+0.1 (-3.5 to +3.6)	-0.9 (-4.2 to +2.5)	-0.3 (-3.6 to +3.0)
PWV (m/sec)			
First tertile	Ref.	Ref.	Ref.
Second tertile	-1.8 (-9.1 to +5.4)	-3.7 (-10.5 to +3.0)	-2.7 (-8.8 to +3.3)
Third tertile	+4.4 (-2.7 to +11.5)	+2.5 (-4.1 to +9.1)	+2.1 (-3.9 to +8.0)
ABI[b]			
First tertile	Ref.	Ref.	Ref.
Second tertile	-0.9 (-2.1 to +4.0)	+1.2 (-1.9 to +4.2)	0.0 (-2.9 to +2.9)
Third tertile	+2.9 (-0.2 to +5.9)	+3.2 (+0.2 to +6.2)	+1.5 (-1.4 to +4.5)

[a] Model 0 = unadjusted; Model 1 = adjusted for sex and age; Model 2 = adjusted for sex, age, smoking status, BMI, SBP, total cholesterol, HDL, TG, and DM. [b] ABI differences were not adjusted for SBP, as it is already included in ABI itself.

In previous studies, increased hepcidin has been associated with increased arterial stiffness (PWV) in patients on maintenance hemodialyis [115], as well as with the presence of carotid plaques in patients with nonalcoholic fatty liver disease and metabolic syndrome alterations [116, 117].

In the past, two studies investigated the relation between blood donation and subclinical atherosclerosis [49, 51]. Zheng and colleagues [49] found decreased oxidative stress and enhanced vascular function in high-frequency donors compared to low-frequency donors, and Engberink and colleagues [51] concluded that IMT was only slightly reduced in high-frequency donors compared to low-frequency donors. So, both studies did not entirely rule out a beneficial effect of frequent blood donation on atherosclerosis, whereas our study does. These previous studies used non-sex-specific categorization of high- and low-frequency donors. As men are allowed to donate more often than women, this could lead to imprecise cutoff values of the determinant under study (i.e., number of donations). In contrast, we created sex-specific cutoff values for lifetime number of whole-blood donations, thereby taking into account sex differences in number of donations.

Health studies among blood donors are very susceptible to a type of selection bias, which is called the healthy donor effect. Recent work from our research group showed that these studies are very susceptible to two types of healthy donor effects [53]. First, during the registration process of new blood donors, health- and lifestyle-related criteria are applied. As a result, the donor population might on average be healthier than the general population.

Second, during the course of a donor's career, the selection process continues as deferral criteria such as repeatedly low Hb levels, hypertension, and cardiovascular disease are applied before each donation. Subsequently, deferred (lapsed) donors might be less healthy than current (active) donors. In contrast, results showed that the healthy donor effect was minimal between active low- and high-frequency donors.

Therefore, to diminish the healthy donor effect, health studies among blood donors should be embedded within a group of active donors [53]. Although Engberink and colleagues mainly compared current donors to ex-donors and never donors, they also performed their analyses within current donors comparing high-frequency donors to low-frequency donors [51]. They found that high-frequency donors had a non-significantly lower carotid IMT than low-frequency donors. However, when excluding plasma donors, the mean carotid IMT increased in high-frequency donors. In contrast, Zheng and colleagues only included active donors in their study and found a strong effect even in quite a small study population [49]. Of note, as a result of their sample size, they were unable to adjust for all confounding factors at once.

In our study we did not exclude lapsed donors, but we did perform a post hoc analysis among currently active donors (at least one donation in the past 2 years, n = 99), with the same adjustments for confounding variables except diabetes mellitus, as this condition is a deferral criterion for blood donation. The results of these analyses confirmed those found in the entire study popu- lation; the maximum difference in IMT, PWV, or ABI between donors in the third sex-specific donation tertile compared to those in the first was 3.6% (data not shown). Thus, we still did not find donors in the third sex-specific donation tertile to be healthier with respect to subclinical atherosclerosis. The healthy donor effect therefore could not have influenced our results, which strengthens our findings of no substantial effect of lifetime number of whole-blood donations on the extent of subclinical atherosclerosis.

Remarks must be made about the implications of our results with regard to the outcome measurements used in this study. Although measurements of subclinical atherosclerosis (IMT, PWV, and ABI) are quite predictive for future cardiovascular disease events [118–121], they are biomarkers and should be interpreted as such. Any effects of blood donation on manifest cardiovascular disease can thus not be determined in our study. We can only draw conclusions about the effects of blood donation on atherosclerosis. Moreover, pathways leading from blood donation to cardiovascular disease other than atherosclerosis, for example, cardiac arrhythmia, were not addressed in this study. Therefore, we cannot entirely exclude an effect of blood donation on cardiovascular disease.

A limitation of our study is the time lag between the actual exposure (i.e., donating blood) and measuring the outcome. This time lag may have weakened any association, if present. It could also have had its influence on the amount of exposure measured in our population. A second limitation is the proportion of iron-depleted subjects within this group of donors. It is questionable whether 6.8% of men and 20.0% of women being iron depleted within the third donation tertile is adequate enough to reveal a protective effect on cardiovascular risk.

With regard to hepcidin, values in donors within the second and third sex-specific donation tertile are quite comparable to those found in the general population [68], which implies nonadequate iron depletion in our study sample. In our post hoc analyses among active donors we were able to investigate the impact of these two limitations. Because these analyses only included currently active donors, there was no time lag as the time since their last exposure (donation) was at the maximum of 2 years.

Furthermore, these donors were also more iron depleted (15.0% of men and 33.3% of women within the third donation tertile compared to 0.0 and 11.1% in the first donation tertile, respectively). Because our post hoc analyses did not deviate from the null results found in the entire study population, these limitations cannot explain the lack of association between lifetime number of donations and subclinical atherosclerosis.

In conclusion, this study showed that blood donation leads to decreased iron stores, but that increased blood donation is not evidently associated with less subclinical atherosclerosis as measured with IMT, PWV, and ABI. The discordance between our results and those from previous studies on blood donation and subclinical atherosclerosis warrants further detailed research. Future studies on cardiovascular disease in whole-blood donors should use sex-specific donation groups and should be embedded within the active donor population.

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Competing interests

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to TRANSFUSION.

Chapter 4

Healthy Donor Effect

The healthy donor effect (HDE) is a type of selection bias occurring in observational studies that is introduced by the repeated health screening and subsequent selection of healthier blood donors. So far, we have minimized the HDE by comparing high-frequency long-career blood donors to low-frequency short-career blood donors, but when the initial analyses on cardiovascular morbidity and mortality were conducted [Chapter 5], this comparison appeared to leave room for the HDE: remaining a donor and continuing to donate automatically implied a better health. This phenomenon of healthy donor selection makes it quite difficult to make any causality claims from observational studies, and requires a new approach to avoid the association between disease-free survival and donation frequency that is not merely a result of healthy donor selection.

Meanwhile, the concept of blood donation was further decomposed into two main assets: the duration of the donation career, and the donation frequency. It was assumed that the effects of blood donation on cardiovascular morbidity and mortality were not of acute nature. Rather, continuing blood donation for at least ten years was considered a sufficient amount of time for blood donation to exert its potential beneficial effect on cardiovascular disease occurrence.

At first glance, restricting the inclusion to donors with a donation career of at least ten years seems a good approach to appreciate both the HDE-bias and the biologically required duration of exposure. Using simulation models, this chapter explores whether the application of a 10-year qualification period will minimize the Healthy Donor Effect without completely diminishing the underlying causal effect of blood donation on cardiovascular events.

4.1 Simulation Study HDE

Original manuscript title:
The qualification period to de-bias the Healthy Donor Effect: a simulation study.
To be submitted

BACKGROUND: High-frequency blood donation has been associated with a reduced cardiovascular risk. However, blood donors are repeatedly selected on health status throughout the donor career, resulting in a seemingly protective effect of blood donation that is actually explained by this Healthy Donor Effect. Instead of using the lifetime number of donations, this study tests whether a qualification period in which high- and low-frequency donors qualify themselves prior to the follow-up period, yields less biased results.
METHODS: A cohort of 1,000,000 simulated persons lowered iron levels with each donation. CVD-risk increased based on iron levels and lifestyle, representing all other causal risk factors. Transition probabilities from non-donor to active donor, from active to stopped donor, and the probability of donating were either completely random or dependent on the CVD-risk. Both simulation scenarios (with a random or healthy donor selection) were analysed with the conventional and a qualification period of 1, 5, 10, and 15 years. Cox proportional hazards modelling estimated the crude and lifestyle-adjusted hazard rate ratio (HRRs) for high- vs. low-frequency donors.
RESULTS: The conventional approach severely overestimated the protective effect of blood donation, with HRR=0.67 even when no causal effect was simulated and donors were randomly selected. When a small causal effect was simulated and healthy donors were selected, the HRR with the conventional approach was 0.16 whereas the 1-, 5-, 10-, 15-, and 20-year qualification period approaches showed HRRs of 0.83, 0.78, 0.32, 0.16, and 0.17. The magnitude of the HDE increased with longer qualification periods and a larger causal effect of iron levels on CVD-risk. Adjusting for lifestyle attenuated the HRRs only in the scenario of a healthy donor selection, and especially when the causal effect of iron levels was relatively small and longer qualification periods were applied.
CONCLUSION: The application of a qualification period during which donors qualify themselves decreases the Healthy Donor Effect, especially compared to the conventional approach using lifetime number of donations. Although some bias will be left unaccounted for, the qualification period is a promising next step in eliminating the HDE-bias.

Introduction

Iron depletion has long been hypothesised to have protective effects on cardiovascular risk. As a result of repeated erythrocyte iron loss, blood donors have depleted iron stores. Not surprisingly, blood donors have been used extensively to study the effect of iron depletion in relation to disease occurrence. Donors have been compared to non-donors, ex-donors, or to donors with a different donation exposure to study the effect of low iron stores and blood levels on cardiovascular disease occurrence. However, results from such studies can be tremendously biased due to the Healthy Donor Effect, a phenomenon resulting from selection processes before and during a donor's career. The Healthy Donor Effect (HDE) comprises factors (e.g. blood pressure, genetic susceptibility, Hb level) that are causally associated with both exposure and disease status, thus influencing the study's estimates of association between blood donation and (cardiovascular) outcome.

Three types of HDE

Atsma et al. previously described three distinct types of the HDE and referred to them as the Healthy Registration Effect (HRE), the Healthy Donor Survivor Effect (HDSE), and the Healthy Donor Career Effect (HDCE) [Figure 4.1] [53]. The HRE distorts research that compares donors to non-donors. When someone applies for blood donorship, a number of health criteria have to be met in order to be registered as a blood donor. This selection process based on (underlying) disease risk is thus responsible for the selection bias when subsequently comparing the health of donors to non-donors. Once being a newly registered blood donor, donation can only take place if certain health criteria are met. Some of them result in a temporary deferral; others mean the end of a donor's donation career. Consequently, currently active donors are more likely to be healthier than lapsing- or stopped donors. Research comparing these two groups of donors is therefore influenced by the HDSE. The third type of HDE, the HDCE, is of importance when studying health effects of blood donation within currently active donors. As a result of continuously applying health criteria prior to each donation, high-intensity donors and those with a higher number of lifetime donations are probably healthier than donors who are yet in an early phase of their donation career. After comparing self-reported health and lifestyle indicators between all donor groups, Atsma and colleagues concluded that it is best to only make comparisons within the active donor population, as the HDCE constitutes the smallest bias [53]. Long-career (high-frequency) donors more often reported a good or excellent self-rated health, and less often a visit to the GP in the previous 3 months than short-career (low-frequency) donors. Although lifestyle indicators such as dietary patterns and physical activity did not materially differ between these two donor groups, BMI was higher in long-career donors than in short-career donors [53].

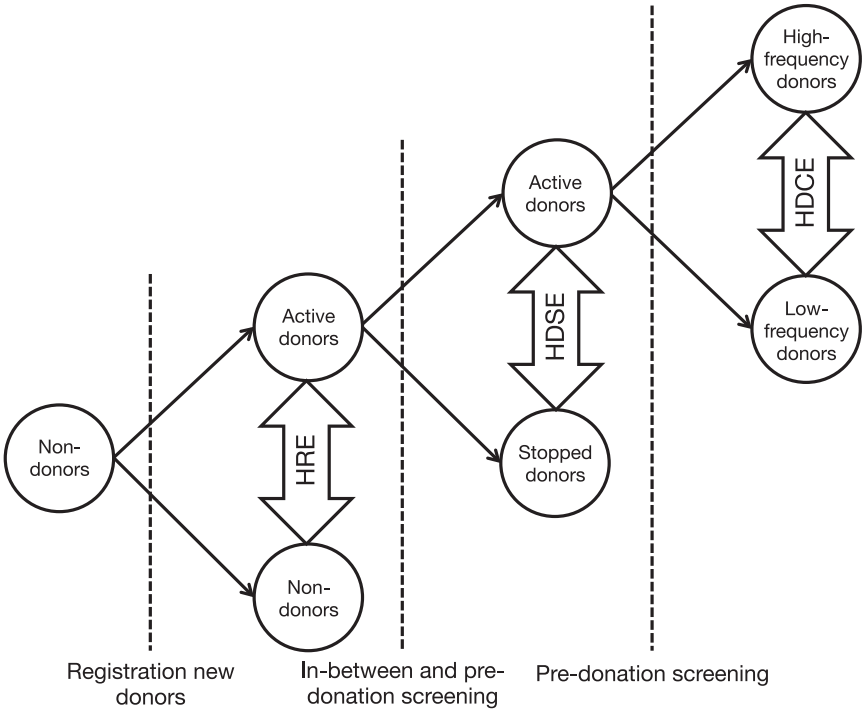


Figure 4.1: Three different types of the HDE (arrows) as a result of three different selection moments (dotted lines) during the donation career. HRE, healthy registration effect; HDSE, healthy donor survivor effect; HDCE, healthy donor career effect.

Epidemiological concepts

Although all three types of the Healthy Donor Effect are referred to as selection bias (a differential selection that occurs before exposure and disease) or confounding (a risk factor for the disease that is also associated with the exposure of interest), once these factors responsible for selection (and confounding) are measured on all study subjects, and these factors are antecedents of both exposure and disease, adjustment just like with any other ordinary confounding factor can be performed according to Rothman, Greenland and Lash [122]:

> Selection bias and confounding are two concepts that, depending on terminology, often overlap. For example, in cohort studies, biases resulting from differential selection at start of follow-up are often called selection bias, but in our terminology they are examples of confounding. Consider a cohort study comparing mortality from cardiovascular diseases among longshoremen and office workers. If physically fit individuals self-select into longshoreman work, we should expect longshoremen to have lower cardiovascular mortality than that of office workers, even if working as a longshoreman has no effect on cardiovascular mortality. As a consequence, the crude estimate from such a study could not be considered a valid estimate of the effect of longshoreman work relatie to office work on cardiovascular mortality.
>
> Suppose, however, that the fitness of an individual who becomes a longshoreman could be measured and compared with the fitness of the office workers. If such a measurement were done accurately on all subjects, the difference in fitness could be controlled in the analysis. Thus, the selection effect would be removed by control of the confounders responsible for the bias. Although the bias results from selection of persons for the cohorts, it is in fact a form of confounding.

However, it is often impossible to exactly know, let alone measure, these factors responsible for the Healthy Donor Effect. Moreover, these factors are also intermediary determinants of exposure and disease, meaning that they are not only caused or influenced by exposure, but also affect the probability of subsequent exposure.

If these factors would have been measured accurately on all subjects, it is still not possible to adjust for them by conditional risk estimation, as adjusting for intermediary variables results in over-adjustment bias (the causal effect of the exposure on disease status is completely accounted for by the intermediary variables, leaving a null-effect). It is therefore of utmost importance that analysing data possibly influenced by the HDE is conducted by design and analytical techniques that are as little as possible affected by it.

Study designs

To decrease the HDE as much as possible, it is recommended to embed studies within the active donor population and create the determinant contrast by the "amount" or level of donation [53]. There are several aspects that determine the level of exposure to blood donation:

1. donation frequency or the (lifetime) number of donations
2. donation career in years
3. the average donation frequency per year
4. the combination of 2 and 3 as a measure of donation intensity.

With cardiovascular events as outcome, it is likely that many donors have stopped donating actively, and that comparing the donation frequency between CVD cases and non-cases would actually mean comparing stopped to active donors. Furthermore, is is expected that blood donation would have a lagged effect on cardiovascular disease. Blood donation should therefore be given a sufficient amount of time to slow down the disease process.

As the HDE is the result of repeatedly selecting healthier (prospective) donors throughout the donation career, analysing techniques accounting for follow-up time could be more resilient to HDE. One could think of Cox proportional hazards (Cox PH) modelling and repeated measures techniques (Generalized Estimating Equations), which do not compare cumulative incidences or risks among exposure groups but rather compare incidence rates. However, the survival probability is likely to be highly associated with exposure status, as the number of blood donations is a time-varying exposure. High-frequency donors would therefore probably have the largest disease-free survival; otherwise they wouldn't have been able to become a high-frequency donor.

Others have also struggled with the HDE. In an attempt to minimize this, Meyers and colleagues have used a 3-year period from 1988 until 1990 during which frequent ($\geq$1 donation each year) and casual (1 donation in 3 years) donors were identified [47]. Although cases and controls were matched on sex and birth year, they did not address possible differences in donation career. In a study on blood donation and cancer, Edgren and colleagues have used a case-control approach in which cases of cancer were matched on time with controls: each time a donor was identified as cancer patient, ten random control donors were selected who were alive and disease free at the index date [123]. This method is called incidence density sampling and the calculated odds ratio directly estimates the incidence rate ratio [124]. Although a major improvement in study design in terms of HDE, it is a cumbersome method that does not account for differences in donation career and also requires the use of a latency period to eliminate the HDE-bias (or more specifically reverse-causation bias in this case), the successfulness of which remains to be seen.

The same research group has recently made a new attempt to adjust for the HDE by only considering mortality among "retired" donors (i.e. those with a last donation at ages 64.5-65 years) who survived at least 2 years after their most recent donation (thereby further reducing short-term causal effects of donation) [125]. Including an interaction between the variables donation rate (i.e. average number of donations per year in the preceding 5-year window) and an indicator for ongoing donation (i.e. not yet "retired") in the Poisson model yielded the HDE-adjusted effect for donation rate among non-"retired" donors. However, they could not confirm that their HDE-adjusted protective effect of blood donation was indeed not biased by a residual HDE, as the adjustment factor was estimated among elderly donors.

In this article, we propose an easy-to-use method as a possible solution to separate the period in which the exposure is determined from the period in which disease occurrence takes place, that simultaneously does justice to the hypothesised lagged effect of blood donation. This can be achieved by the application of a so-called qualification period [126]. Although invented for clinical trial settings, we use the qualification period as a fixed period of exposure during which donors must qualify themselves. This means that donors must remain an active donor for at least, say, 10 years [Figure 4.2]. During this qualification period, the exposure is determined (i.e. number of donations). Only after this qualification period, the actual follow-up period starts in which donors can experience cardiovascular events. By fixing the period in which exposure is determined, exposure status cannot have influenced the survival probability as they are now separated periods. Of note, such an approach seems only appropriate in situations in which the exposure-disease relationship is likely to be long, meaning that more acute-onset diseases would be inappropriately studied in this manner.

Aim

This simulation study examines whether a qualification period to determine donation frequency yields better, less biased, results than without a qualification period using the lifetime number of donations (at the end of follow-up) to compare cardiovascular risk between high- and low-frequency donors using Cox PH regression. For this purpose, datasets were created with a completely random donor selection and a healthy donor selection, and subsequently analysed with both the conventional lifetime number of donations method and the use of a qualification period. This was practised on datasets that mimicked a protective effect of blood donation on cardiovascular disease through the lowering of ferritin (as a measurement of body iron stores).

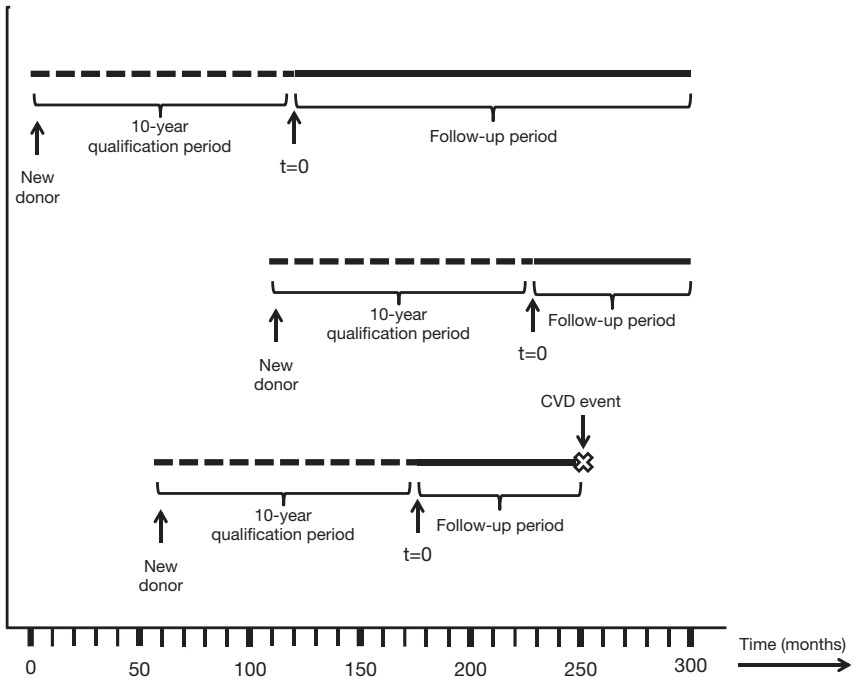


Figure 4.2: Design of a cohort study to which a 10-year qualification period is applied. Inclusion criterion is remaining an active donor for at least 10 years. Donation frequency is determined during this qualification period, whereas disease occurrence is measured during the follow-up period starting after 10 years. The time-axis could be either read as calendar months from fictive years or the iterations used in this simulation study.

Materials and Methods

General model

We simulated a cohort of blood donors that lowered their cardiovascular disease (CVD) risk by lowering ferritin after each donation. Figure 4.3 provides an overview of the model with two of the simulated scenarios. A dataset of 1,000,000 subjects was created, of which 10% was immediately assigned blood donorship. As blood donors are a dynamic population, the constant influx of new donors and efflux of stopping donors was mimicked as well. Therefore, we used a looping procedure, with 300 iterations, each representing one month. In each iteration, there was a constant transition probability from the non-donor to the active donor state, and from active donor to stopped-donor.

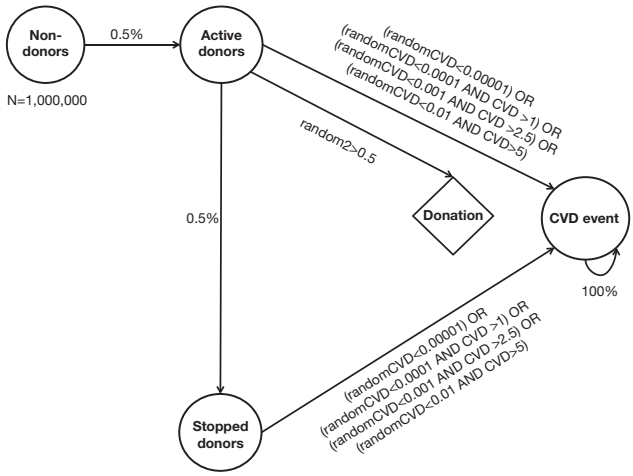
Donors were allowed to donate, whereas non-donors were obviously not. Each subject started with a random ferritin value drawn from a normal distribution with a mean of 100 μg/l and a standard deviation of 20. After a donation, ferritin dropped with 10. When ferritin dropped to levels below 10, the value was set at 10. All donors were first-time donors; they all had 0 previous donations. All donors (active and stopped) increased their ferritin with 1 each month.

CVD events occurred depending on the CVD-level, which started with a baseline CVD level and from thereon increased each month with a fraction of the current ferritin level and a fraction of "lifestyle", representing all other risk factors of CVD. All subjects had a baseline CVD risk randomly drawn from a uniform distribution with minimum 0 and maximum 1, except those immediately assigned blood donorship; their distribution ranged from 0 to 0.5. A higher event probability was assigned to higher levels of CVD. The looping ended when donors experienced a CVD event, or after the 300^{th} iteration.

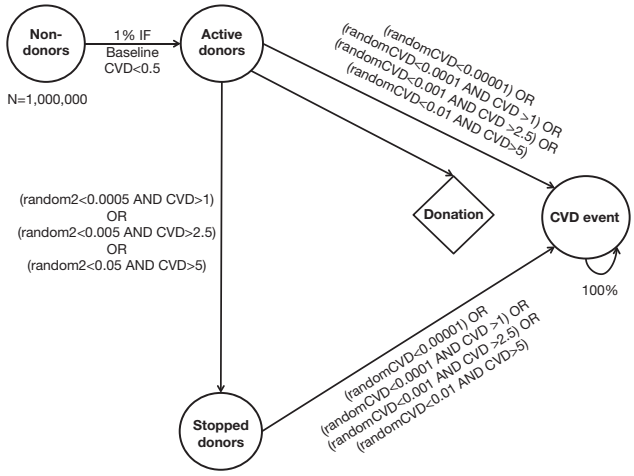
Simulated scenarios

To study the effect of both the conventional approach and the qualification period applied to the data, we used two different scenarios: one with a random donor selection throughout the donor career, and one with a healthy donor selection. CVD-level affected the probabilities of becoming a donor, making a donation, and stopping in the scenario of a healthy donor selection, whereas these probabilities were randomly assigned in the random donor selection scenario.

Simulating the Healthy Registration Effect (HRE) The HRE is a result of selecting healthy prospective donors, and only allowing new donors with a low baseline CVD risk mimicked this effect. For each prospective donor, a random baseline CVD risk was drawn from a uniform distribution with minimum 0 and maximum 1. Only prospective donors with a baseline CVD risk <0.5 were allowed to enter the active donor pool.



(a) Random donor selection.



(b) Healthy donor selection.

Figure 4.3: Schematic overview of the 2 scenarios for donor selection: random or healthy. Combined with 3 scenarios for the size of the causal effect of ferritin on CVD risk: none: $CVD = CVD + (0.0000 * Ferritin) + (0.0002 * Lifestyle)$, small: $CVD = CVD + (0.0005 * Ferritin) + (0.0002 * Lifestyle)$, and large: $CVD = CVD + (0.001 * Ferritin) + (0.0001 * Lifestyle)$, yields $2 * 3 = 6$ scenarios.

Simulating the Healthy Donor Survivor Effect (HDSE) In the scenario of random donor selection, there is a probability of 1% to change in donor status from active to stopped, irrespective of CVD risk. Subsequently, HDSE was introduced by the longitudinal CVD risk affecting this probability of continuing blood donorship, with a higher CVD risk increasing the stopping probability.

Simulating the Healthy Donor Career Effect (HDCE) To simulate the HDCE resulting from differential donor selection prior to each donation, donors with a higher CVD-risk had a lower chance to donate: 50% for donors with CVD <2.5 and 25% for donors with a CVD <5. This means that only the healthiest ones were able to reach a high number of donations.

Causal effect size To examine whether the size of the causal effect of ferritin on CVD risk changed the results, and to demonstrate that the 10-year qualification did not wash out the entire causal effect, we have used three different causal effects scenarios: (I) no causal effect of ferritin on CVD (using a completely random variable that mimicked the behaviour of ferritin, but was entirely unrelated to blood donation), (II) a small causal effect of ferritin on CVD risk (with an increase of $0.0005 * ferritin$ and $0.0002 * lifestyle$ each month), and (III) a large causal effect of ferritin on CVD risk (with an increase of $0.001 * ferritin$ and $0.0001 * lifestyle$ each month).

Each of the three different causal effect scenarios (none, small, large) also had two different donor selection scenarios (random or healthy). This yields a total of six different simulated scenarios. The complete program can be found in Appendix B.

Statistical analysis

To each dataset, both the conventional method and the qualification period were applied to compare cardiovascular risk between high- and low-frequency donors. For the conventional method, the lifetime number of donations (i.e. at the end of follow-up) was used to distinguish high-frequency donors from low-frequency donors as the exposure measurement, based on the median.

The qualification period approach only selected donors who had remained throughout the entire qualification period. The number of donations that they made during this qualification period was used to categorize donors into low- and high-frequency donors, based on the median as well. Five different durations of the qualification period were used: 1 year, 5 years, 10 years, 15 years, and 20 years. Follow-up time was calculated by subtracting the duration of the qualification period from the total follow-up time between becoming an active donor and having a CVD event or the end of follow-up after 300 months. As a result, they could only have a maximum survival of 288, 240, 180, 120, or 60 months after a qualification period of 1, 5, 10, 15, or 20 years, respectively.

Unconditional and conditional on lifestyle Cox PH models were built to estimate hazard rate ratios (HRRs), with the time until cardiovascular event or for censored donors today's date (t=300) in months as person-time. All datasets were built and analysed with IBM PASW SPSS 21 for Mac (Release Version 21.0.0.0, Chicago, IL, USA).

Results

Overall effects of scenarios

A brief description of the population characteristics is provided in Table 4.1. In the scenario without a causal effect of ferritin on CVD risk, the sole effect of the healthy donor selection can be seen. Because the probability to stop donating is now dependent on the CVD risk, it takes longer for donors to reach a certain CVD level that ends their donor career. As a result, the donor career is longer with a healthy donor selection [Table 4.1]. Furthermore, the healthy donor selection has prevented donors to donate as often as in the scenario of a random donor selection, reflected by a lower number of donations and a higher ferritin level.

Within the scenario of a random donor selection, different causal effects of ferritin on CVD risk only results in different levels of ferritin and the percentage of donors with a CVD event. Although the number of donations does not seem to be affected by the causal effect size of ferritin, ferritin itself is approximately 1.5 times higher when it *does* contribute to CVD risk. This could be a result of CVD-cases having a higher ferritin level in the scenario with a causal effect of ferritin than in the scenario without such a causal effect of ferritin. Another interesting effect of increasing the causal effect of ferritin, seems to be the increased proportion of CVD-cases. One could expect that more benefit from donating blood is gained when the causal effect of ferritin is increased. Instead, the CVD-risk increases more rapidly when ferritin has a larger causal effect (0.001*ferritin vs. 0.0005*ferritin), irrespective of blood donation. Thus, the proportion of CVD-cases is around 1.3 times higher in the scenario of a large causal effect of ferritin on CVD risk.

Independent of the causal effect, the donor career was longer when a healthy donor selection was simulated. However, when ferritin did contribute to CVD risk, more donors remained active when a healthy donor selection was applied, likely a result of lowering their CVD risk with blood donation. This preventive effect of donating blood was especially present in the scenario of a small causal effect of ferritin, because the healthy donor selection differentiates quicker between those at a low or high CVD risk when the causal effect is larger: not being able to donate when your CVD risk is higher results in a more rapid increase of your CVD risk, with earlier drop-outs. Compared to a small causal effect of ferritin, this stronger or more rapid selection effect when a large causal effect is simulated is also reflected by a shorter donation career with fewer donations [Table 4.1].

Table 4.1: Characteristics of simulated donor cohorts. Data are reported as median or percentage

	Random donor selection	**Healthy donor selection**
	No causal effect of ferritin on CVD risk	
Donor status		
Non-donor	22%	22%
Active	38%	35%
Stopped	40%	43%
Donation career (months)	100	115
Number of donations	46	36
Ferritin (μg/l)	27	40
CVD event	35%	35%
	Small causal effect of ferritin on CVD risk	
Donor status		
Non-donor	22%	22%
Active	35%	50%
Stopped	43%	28%
Donation career (months)	99	158
Number of donations	49	61
Ferritin (μg/l)	44	14
CVD event	29%	20%
	Large causal effect of ferritin on CVD risk	
Donor status		
Non-donor	22%	22%
Active	36%	38%
Stopped	42%	40%
Donation career (months)	100	123
Number of donations	47	40
Ferritin (μg/l)	38	39
CVD event	37%	37%

Dynamic donor populations are simulated in which blood donation lowers ferritin and donors develop CVD events. Six different donor cohorts are created with 2 scenarios of donor selection (random and healthy) and 3 scenarios of causal effect of ferritin on CVD risk (none, small, and large).

Effect of analysing techniques

In the scenario without a causal effect of ferritin on CVD risk and a random donor selection, all qualification-period approaches yielded HRRs≈1 [Table 4.2], confirming no protective effect of high-frequency blood donation. In contrast, the conventional approach leads to a seemingly protective effect of high-frequency blood donation, with HRR=0.67. When a healthy donor selection is applied to the scenario of no causal effect, the conventional approach further overestimates the protective effect in high-frequency blood donors to a HRR of 0.31, a typical "healthy donor effect". Likewise, the qualification period approach has stronger deviations from 1 when the duration of the qualification period increases; more time during which donors are selected on their CVD-risk [Table 4.2]. Of note, the "healthy donor effect" seems to stabilize around HRR=0.38 from a 10-year qualification period onwards, and does not completely converge to the overestimation as observed in the conventional lifetime approach. This likely stems from a different selection of the donor population: the 20-year qualification approach does not include donors that have failed earlier, whereas the conventional lifetime approach does, leading to a larger overestimation probably by the comparison of stopped and active donors.

The scenarios with a causal effect of ferritin on CVD risk demonstrate a further protective effect in high-frequency blood donors compared to the no causal effect scenario. For the qualification period approaches with a random donor selection, HRR≈0.92 in the scenario of a small causal effect and HRR≈0.84 in the scenario of a large causal effect of ferritin [Table 4.2]. However, in the scenario of a healthy donor selection, the HRRs further decrease to indicate a stronger protective effect in high-frequency donors. Although the HRRs with the conventional lifetime approach do further decrease to reveal the beneficial effect of blood donation in the scenarios with a causal effect of ferritin, the difference between the random and healthy donor selection scenarios are not that clear as all HRRs remain around 0.22 [Table 4.2].

By comparing the HRRs of the scenario with a healthy donor selection to the HRRs of the scenario with a random donor selection, it appears that the healthy donor selection results in an overestimation of the effect of blood donation. This overestimation seems to be affected by the size of the causal effect and the duration of the qualification period. The overestimation is larger when the causal effect is large, but decreases when a longer qualification period is applied. This phenomenon can be explained by the vicious circle that is brought upon by the healthy donor selection: a high CVD-risk leads to a lower donation probability, thus increasing the CVD-risk. Therefore, a larger causal effect results in a bigger overestimation of the protective effect of blood donation, but this becomes less important when a longer qualification period is applied. If a longer qualification period is applied, other factors such as lifestyle become more important in determining CVD-risk. This can be observed by comparing the lifestyle-adjusted HRRs within the scenario of a healthy donor selection between the different qualification periods: the confounding effect of lifestyle is larger when the qualification period is longer.

Table 4.2: Effect of analysing scenario on estimated Hazard Rate Ratio (HRR) of high- vs. low-frequency donors on cardiovascular events

		Simulation scenarios					
		No causal effect		Small causal effect		Large causal effect	
	Adjusted for life-style	Random donor selection	Healthy donor selection	Random donor selection	Healthy donor selection	Random donor selection	Healthy donor selection
Analysing scenarios				1-year qualification approach			
	No	1.00	1.01	0.90	0.83	0.83	0.63
	Yes	1.00	1.01	0.89	0.82	0.83	0.63
				5-year qualification approach			
	No	1.00	0.45	0.92	0.78	0.85	0.43
	Yes	1.00	0.53	0.92	0.82	0.85	0.45
				10-year qualification approach			
	No	1.00	0.38	0.92	0.32	0.84	0.38
	Yes	0.99	0.50	0.91	0.67	0.83	0.43
				15-year qualification approach			
	No	0.99	0.37	0.93	0.16	0.82	0.33
	Yes	0.98	0.49	0.92	0.54	0.81	0.40
				20-year qualification approach			
	No	1.02	0.38	0.94	0.17	0.85	0.32
	Yes	1.01	0.49	0.94	0.63	0.83	0.38
				Conventional lifetime approach			
	No	0.67*	0.31	0.23	0.16	0.22	0.21
	Yes	0.67*	0.37	0.23	0.26	0.22	0.21

* The conventional lifetime approach uses the entire donor population and the total lifetime number of donations to categorise high- and low-frequency donors. Consequently, donors with a very short follow-up time will inevitably be categorised as a low-frequency donor, and this also works the other way around: high-frequency donors will have a longer follow-up time. This association between donation frequency and disease-free survival creates a seemingly protective effect of HRR=0.67. In contrast, the qualification period approach uses a fixed period of exposure during which donation frequency is determined. This number of donations during the qualification period is used to categorise high- and low-frequency donors. The period during which exposure (i.e. donation frequency) is determined is thus separated from the occurrence of CVD events, as only donors that have survived the entire qualification period as an active donor are selected.

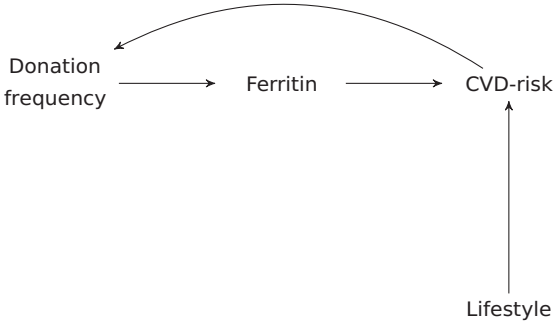


Figure 4.4: Causal diagram with a causal effect of ferritin on CVD-risk. Each donation lowers ferritin levels, which affects CVD-risk along with Lifestyle. CVD-risk determines the donation probability, reflecting a healthy donor selection responsible for the HDE-bias.

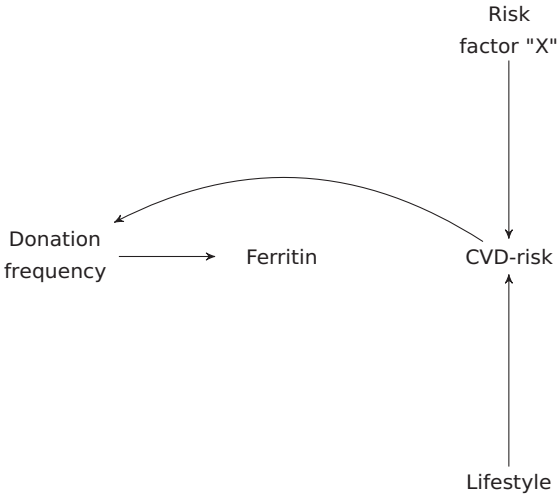


Figure 4.5: Causal diagram with no causal effect of ferritin on CVD-risk. Risk factor "X" denotes a random risk factor of CVD that mimicks the behaviour of ferritin in size and change but is entirely independent of blood donation. Ferritin does not affect CVD-risk in this scenario. Lifestyle operates as a confounding variable by indirectly influencing the donation probability via CVD-risk.

Lifestyle as a confounder?

Lifestyle only seems to act as a confounder of the relation between blood donation and cardiovascular events if a healthy donor selection is present. This is a result of CVD-risk determining donation frequency when a healthy donor selection is simulated [Figure 4.4]. The presence of "confounding" even in the scenario of no causal effect of ferritin, reveals how lifestyle and donation frequency must be associated through CVD-risk [Figure 4.5], as no direct effect of lifestyle on donation frequency was modelled. Lifestyle is (indirectly) associated with donation frequency (through CVD-risk) and a direct cause of CVD-risk, therefore acting as a confounder.

Because the healthy donor selection creates a cyclic causal diagram, lifestyle cannot be considered a confounding factor in the classical sense, but merely acts like one through CVD-risk. Estimating the direct causal effect of donation frequency on CVD-risk is nearly impossible because of the feedback loop of CVD-risk on donation frequency. This simulation shows that adjusting for lifestyle or other independent risk factors for CVD-risk yields better effect estimates, especially when the true causal effect of ferritin is relatively small.

Discussion

This simulation study has shown that when comparing cardiovascular risk between high and low-frequency donors, applying a fixed qualification period that donors must have survived and in which their donation frequency is determined strongly decreases the Healthy Donor Effect (HDE) bias, as compared to the conventional approach of using lifetime number of donations over the entire follow-up period to distinguish high-frequency donors from low-frequency donors. This is especially true for shorter qualification periods and smaller causal effects of ferritin on CVD-risk.

We can now extend the conclusion from Atsma and colleagues that comparisons between high- and low-frequency donors are more robust to the HDE, *if and only if* a qualification period is applied. When the entire follow-up period is used to determine donation frequency, biased effect parameters will be found due to a higher survival probability of high-frequency donors. Consequently, the two different exposure groups will inevitably be selected on outcome/disease status. This simulation study has further proven that even without the selection of healthy donors throughout the donor career, and even without a causal, protective effect of blood donation on CVD risk, one will still find a seemingly protective effect of high-frequency blood donation compared with low-frequency blood donation with this conventional approach.

This "time-effect" of high-frequency donors having a longer disease-free survival (otherwise they could not have become a high-frequency donor), independent of a protective effect of blood donation, is not seen in the qualification period approach. We have confirmed that determining the exposure frequency of donation prior to the outcome, efficiently separates most of the selection processes that occur during follow-up. However, factors that determine the donation frequency during the qualification period, and that transfer across the qualifica-

tion period to the follow-up period, still bias the risk estimates. The healthy donor selection that takes place during the qualification period will therefore impose stronger biases when the qualification period is longer.

Depending on the magnitude of the causal effect of blood donation and ferritin on CVD-risk relative to other risk factors of CVD, the healthy donor selection result in a smaller or larger bias. In general, the overestimation of the protective effect of high-frequency blood donation due to healthy donor selection, is larger when the causal effect is larger. The divergence between high- and low-frequency donors will be quicker when the health benefit of blood donation (i.e. a larger causal effect) increases; donors not being able to donate due to a higher CVD-risk more rapidly fall behind in donation frequency and drop out of the donor population. This "positive feedback-loop" or vicious circle thus creates a stronger HDE when there is more to gain with blood donation, but when longer qualification periods are applied other causal risk factors of CVD such as lifestyle become more important. Therefore, the magnitude of the HDE with the qualification period approach is larger when a longer qualification period is used (more time during which donors are selected) and the causal effect of blood donation is smaller (leaving more room for other causal risk factors such as lifestyle).

The most valid estimates are obtained in the qualification period approach, using a period of 1 year. The subsequent question would be whether such an approach is the right one in a situation in which the effects of blood donation are assumed to be lagged. Lowering iron stores for 1 year with frequent blood donation can hardly be assumed to have causal protective effects on cardiovascular disease, because this disease has a longer time to develop. Longer qualification periods will thus be required from a biologic perspective, but increases the size of the HDE. Furthermore, the importance of measuring other causal factors to control for them is emphasised by the results of this simulation study. This becomes especially important when the true causal effect of blood donation is small.

The difficulty of the HDE is the simultaneous ability of factors to confound and being part of the causal pathway between exposure and outcome. There are other, more advanced statistical approaches such as g-estimation and marginal structural models (MSMs) that would ordinarily be applied in such circumstances. However, they cannot be used in this context. The strict exclusion of donors that do not meet the criteria for blood donation (a probability to donate of zero), violates one of the important assumptions of MSM: the positivity assumption. This assumption dictates a probability of > 0 for each exposure stratum. Of note, this simulation study has not used such strict exclusion criteria, but simply decreased the probability of a donation for donors with a higher CVD-risk. Although we could have used the advanced statistical modelling techniques to our simulated data, they would not do just to the true observed blood bank data in which donors with a blood pressure above a certain threshold have a probability of zero to donate.

Another deviation of our simulated data with real-world data, is the omission of using ferritin (or more precisely haemoglobin) as a pre-donation criterion. A deferral for a donation because of low haemoglobin levels is not uncommon; approximately 26.7% of the active

donor population has had at least one deferral [Section 2.2]. Of particular interest, is the direction of the HDE bias by Hb-deferrals. This would allow donors with higher iron levels to donate more often, thus enabling 'unhealthier' donors to become high-frequency donors. Depending on the magnitude of the two gross selection mechanisms of CVD-risk (healthier) and iron levels (unhealthier), the size of the HDE bias will likely vary. This simulation study has solely focussed on selecting healthier donors, mainly to investigate its plausibility of explaining beneficial cardiovascular health effects in previous observational studies. In addition, lifestyle was only modelled as a direct contributing factor of CVD risk through which it was also related to donation frequency, whereas in reality it is likely to be also a direct causal factor of donation frequency. As with all pre-donation selection criteria, lifestyle constitutes several aspects with varying effects on the direction of the HDE bias. The main and sole purpose of lifestyle in this simulation study was to incorporate other causal factors of CVD than ferritin, for example dietary pattern and physical activity, in order to balance out the different sufficient causal factors that form the causal model of CVD risk.

Even in the absence of a protective effect of frequent blood donation on CVD, and even in the absence of a continuous healthy donor selection, using the lifetime number of donations as exposure measure severely overestimates the "protective effect". Using a short qualification period prior to the outcome period, effectively decreases this healthy donor effect, but seems illogical from a biologic point of view assuming a lagged effect of blood donation. Furthermore, controlling for other causal risk factors of CVD is important as they act as confounders due to the healthy donor selection, especially when the true causal effect of blood donation is small and longer qualification periods are applied. Future studies should be aware of the magnitude of the HDE within donors not only when using lifetime number of donations but also when using a long qualification period to study the effect of frequent blood donation on long-term outcomes such as CVD, cancer or other hypothesised iron-mediated diseases such as Alzheimer's disease.

Chapter 5

Cardiovascular Outcomes

So far, we have sought the answer to our question on the subclinical level, but the proof of the pudding is in eating. What is the magnitude of the preventive ability of blood donation on cardiovascular disease? Are we really able to prevent cardiovascular disease with frequent blood donation? And if so, does blood donation also lower cardiovascular mortality? If there really were to be such a thing as preventing cardiovascular morbidity and mortality through blood donation, what would be the best donation intensity to maximize its preventive strength?

5.1 Morbidity and Mortality

Original manuscript title:
Reducing cardiovascular disease with 10 years of regular blood donation: a cohort study of 159,934 donors addressing the healthy donor effect.
To be submitted

BACKGROUND: Prolonged iron depletion could protect against cardiovascular disease as a result of decreased oxidative stress. Due to repeated erythrocyte loss, frequent blood donation lowers iron stores. This study aims to investigate whether 10 years of active whole-blood donating decreases the risk of cardiovascular mortality and morbidity.
METHODS: All Dutch whole-blood donors who have remained active for at least 10 years (validated by $\geq$ 1 donation in either year 8 or 9) were included in this study. During this 10-year qualification period, donors were categorized into sex-specific donation tertiles according to the total number of whole-blood donations. End-points were cardiovascular cardiovascular morbidity (investigated in 155,827 donors) and mortality (investigated in 158,919 donors) based on hospital discharge diagnoses and death certificates from Statistics Netherlands. Cox-regression was used to estimate the age-adjusted Hazard Rate Ratio (HRR) with a 95% confidence interval (95%-CI).
RESULTS: Median age at start of donation career was 34 (IQR: 16). A total of 9,381 (10.81%) men and 4,338 (6.28%) women had a primary cardiovascular hospital admission or death. Primary cardiovascular mortality was confirmed in 876 (0.55%) donors. Adjusted for age at start of the donation career, female high-frequency blood donors had a reduced cardiovascular morbidity (HRR=0.90, 95%-CI: 0.84 - 0.97) and cardiovascular mortality (HRR=0.83, 95%-CI: 0.56 - 1.22) compared with low-frequency blood donors. No effect was observed in men on either morbidity (HRR=0.99, 95%-CI: 0.94 - 1.04) or mortality (HRR=0.94, 95%-CI: 0.78 - 1.12)
CONCLUSION: Long-term, high frequency blood donation protects against cardiovascular disease in women, but not in men. Follow-up time was insufficient in this young population to statistically confirm effects on cardiovascular mortality.

Introduction

Iron catalyses the formation of reactive oxygen species. These in turn can damage vascular endothelium, pancreatic beta cells, and hepatocytes. Not surprisingly, high levels of iron stores have been repeatedly related to diseases from the cardiovascular spectrum: atherosclerosis, AMI, metabolic syndrome, non-alcoholic fatty liver disease, and diabetes mellitus. However, such associations were at least as often unproven as they were proven [98]. In studies from the general population, high iron levels could also be the consequence of chronic subclinical inflammation. Inflammation is, along with iron level and hypoxia, one of the main mechanisms that affect iron homeostasis through the expression of hepcidin [24]. Thus, reverse causation bias could have largely influenced such studies.

Blood donors are a profoundly eligible group of subjects from the general population that are not only generally healthy and disease-free (otherwise they wouldn't have been able to become a donor), but also have low iron stores. The latter mainly being a result of the repeated loss of whole-blood, containing iron-rich erythrocytes. Comparing the risk of cardiovascular disease between donors and non-donors, although not uncommon in previous studies, would lead to biased results due to a so-called healthy donor effect. It has therefore been recommended to perform studies embedded within the donor population, comparing high-frequency donors to low-frequency donors [53].

To date, a few such studies have been performed and found that high-frequency blood donation is associated with: increased flow mediated dilation [50], decreased carotid intima media thickness [51], increased insulin sensitivity [48], and a lower incidence of cardiac events [47]. However, a quasi-random experiment in blood donors found no effect (a risk ratio of 1.02) of blood donation on incident cardiac ischemia [52]. This null-effect was also confirmed in a recent study among Italian blood donors [127] comparing hospital admissions between donors with different donation frequencies and years of donation to non-donors. In a recent study on blood donation and all-cause mortality, an attempt was made to adjust for the healthy donor effect [125]. Although their HDE-adjusted results indicated a protective effect, they also could not exclude the presence of a residual HDE that explained this result.

Furthermore, the same study by Zheng et al. that found improved flow-mediated dilation in high-frequency blood donors also refuted the hypothesis that changes in glucose metabolism would link blood donation to improved vascular function, as it found no effect on insulin sensitivity or vascular reactivity after oral glucose loading [50]. In line with these results, previous studies from our own research group either found no association between frequent high-intensity blood donation and vascular function [105] or metabolic syndrome prevalence [74], or even a counter-hypothesised increase in insulin resistance in a cohort of first-time donors followed-up for 1.5 - 2 years [Peffer et al, unpublished].

All in all, no definite conclusions can be drawn. Maybe a more composite end-point, such as overt cardiovascular disease, will have a better opportunity to reveal any association with blood donation. Again, the healthy donor effect could be an issue, as such long-term outcomes will frequently occur outside the donation career, and that comparing high-frequency (long-career) donors to low-frequency (short-career) donors would actually mean

comparing healthy active donors with less-healthy stopped donors. Furthermore, for such more long-term health outcomes, we assume that a longer exposure window of 10 years is needed for blood donation to exert its protective effect on cardiovascular disease. By only including donors who have remained active in donating for at least 10 years, and compare those with a high donation frequency during these first 10 years to those with a low donation frequency, we can study the effect of intensive blood donation on long-term outcomes such as cardiovascular disease. Moreover, it excludes the comparison of active to stopped donors as each donor was still active when their donation frequency was determined, thereby theoretically reducing the healthy donor effect.

In a simulation study from our own group, we showed that implementing such a 10-year qualification period further reduced the healthy donor effect as compared to using the lifetime number of donations [Peffer et al, unpublished]. Such a qualification period is also used in more clinical trial settings to eliminate other causal factors [126]. By applying a 10-year qualification period, the disease-free survival time or duration of the donation career is not necessarily related with the number of donations, as each donor has had equal opportunity to donate in this fixed time period. Because the study outcome does not influence the number of donations, the qualification period approach reduces the healthy donor effect between high- and low-frequency donors.

In the present study, we aim to estimate the relation between blood donation and incident cardiovascular disease by including a very large cohort of all Dutch whole-blood donors ever. Even within the donor population, a healthy donor effect exists as the repeated (self-) selection of healthy donors throughout the donation career results in health differences between high- and low-frequency donors that appear to be a result of their donation behaviour, but actually is the result of (self-) selection. By applying a 10-year qualification period during which donors must have remained active in donating, we aim to minimize this healthy donor effect.

Materials and Methods

Data sources

For this study, all electronically recorded donations, including visits that ended in a deferral, thru 2010 were merged. In the early 80s, blood donations were started being recorded electronically by each individual collection centre. As of 1990, the electronic recording of donations had a near nationwide coverage. Since 1998, Sanquin Blood Bank has been the sole foundation that is responsible for blood supply in the Netherlands.

Because several regions have been responsible for the blood collection registry over the years, donors could be registered in several blood banks. During several centralization processes, older blood bank registries were merged into newer ones, with donor IDs from the older ones extended to avoid duplicates in the newer ones. This process has continued up to 2005, in which one national blood donor database was created, assigning completely new donor IDs to each donor.

Donor IDs, from older to newer ones, were linked to each other based on the same extension process that took place in the 90s. This extension method was collection-site-specific and was deduced by us from hand-searching dozens of individual donors based on name, address, date of birth, and gender. Also, we had documentation from two former divisions (covering 50% of The Netherlands) on the exact method used for extending donor IDs.

This linkage process resulted in a database of 28,229,353 donations from assumingly 1,5 million donors. For each donation record, haemoglobin and blood pressure was merged as well, using donor-ID and donation-ID. Missing values gradually decreased over time, from approximately 95% of donations in the 80s having missing Hb and blood pressure values, to 65% and 4% in the 90s and 2000s, respectively. Of note, missing values naturally occur when donors have not been tested because they are deferred for other reasons, e.g. risk behaviour. In later years, body weight and length was also recorded, which we have used to calculate the body-mass-index (kg/m^2). However, these fields are donor-specific instead of donation-specific, and are also overwritten throughout visits. Thus, no real longitudinal data is available for BMI, except over the different databases used over time. Moreover, weight and length are not always recorded; most often only at the very first visit.

Statistics Netherlands linked donation records based on gender, date of birth, ZIP code, number of the house, and a validity date (last donation date) to the GBA (municipal population register). The GBA contains unique national registration numbers as of 1995. Certificates of death are linked to the GBA for all deaths that have occurred in the Netherlands, and are subsequently recoded into ICD-9 (1995) and ICD-10 (1996-2010) coding. Cause(s) of death of Dutch residents who died abroad are commonly not included in the GBA, but date of death is. A maximum mismatch of 1 in the linkage variables date of birth and gender was allowed. Of the 28,229,353 donation records, 25,195,010 (89.3%) were linked, of which 28,279 (0.1%) were linked with 1 mismatch [Figure 5.1]. In total, 3,034,343 (10.7%) donation records were not linked at all, of which 2,611,593 (86.1%) could not be linked due to missing values in the primary linkage variables zip code and number of the house, and the remaining 422,750 (13.9%) donation records due to missing values in the secondary linkage variables date of birth and gender [Figure 5.1].

Study populations

From the donation records that were linked to the GBA, all donors with at least one successful whole-blood donation were selected. A whole-blood donation with a drawn volume of at least 100 ml was considered as being successful. Donations with missing donation type were considered to be whole-blood if: 1) the donor had been given whole-blood previously or later (the latter one mainly being the case in very old records which did not yet contain information on donation type); and 2) if the interval between two successful whole-blood donations was at least 56 days, in concordance with current and past guidelines. Missing drawn volumes from older donations were ignored for deciding whether the donation was successful or not, as this was not recorded from the beginning onwards.

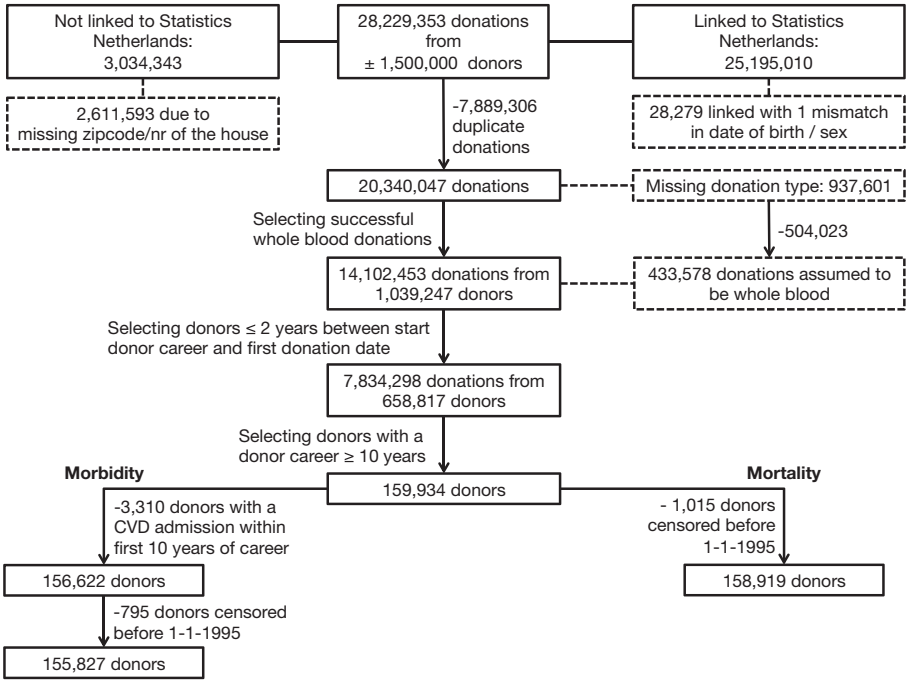


Figure 5.1: Linkage results of the study populations. Donations were linked to Statistics Netherlands (89.3%). Only donors with at least 1 successful whole-blood donation, a completely recorded donation history (≤ 2 years between start donor career and first recorded donation date), and a donor career of at least 10 years were selected.

However, if any previous donation did have a non-missing drawn volume, the donation was regarded as a (temporary) deferral.

In the Netherlands, donors are first invited for a new donor test, a first visit during which blood donation is explained and more extensive medical tests are performed. If all test results are negative and the donor is still willing to become a donor, they are invited for their first donation within 6 months. Because donations were recorded only from a specific, unknown date onwards (left censoring), only donors with a recorded donation maximally 2 years after their first recorded new donor visit or first recorded application date were included. This to ensure that we had the complete donation history of each included donor.

This study uses two different study populations for two different cardiovascular outcomes: morbidity and mortality. In the morbidity study, donors with a CVD hospital admission in the first 10 years of the donor career are excluded. Of the 1,039,247 donors with at least 1 successful whole-blood donation, 159,934 (15%) donors had a complete donation history and had (had) a donor career of at least 10 years [Figure 5.1. This was validated by a successful whole-blood donation in either year 8 or 9 [Figure 5.2]. Our end-points are only available as of 1995. Therefore, donors with a last donation date before 1-1-1995 and who were not known to have had a CVD event and whose deregistration date, if available, was before 1-1-1995, were also excluded.

Morbidity Of the 159,934 donors with a donor career of at least 10 years, 3,310 donors were excluded because of a cardiovascular hospital admission within the first 10 years of the donor career [Figure 5.1]. Another 795 donors censored before 1-1-1995 were excluded, leaving 155,827 donors available for analysis [Figure 5.1].

Mortality From the initial population of 159,934 donors with a donor career of at least 10 years, 1,015 were excluded due to being censored before 1995. This left 158,919 donors available for analysing CVD mortality [Figure 5.1].

Outcome measurements

Morbidity Dutch Hospital Data (DHD) collects hospital admissions and discharges from all hospitals in The Netherlands. For each admission, this registry contains the main diagnosis for which a patient was admitted and subsequently discharged. Recording of main discharge diagnoses was mandatory up to and including 2005 for all hospitals in the Netherlands. Thereafter, coverage slowly declined to 86% in 2010 [128]. Diagnoses are recorded according to ICD-9 coding. The primary outcome for morbidity was the first occurrence of either a cardiovascular main discharge diagnosis or a primary cardiovascular death (ICD-9: codes 3900 - 4599; ICD-10: codes I000 - I999). Because the GBA consists as of 1995, follow-up data on cardiovascular discharges and deaths were available from 1995 through 31st December 2010.

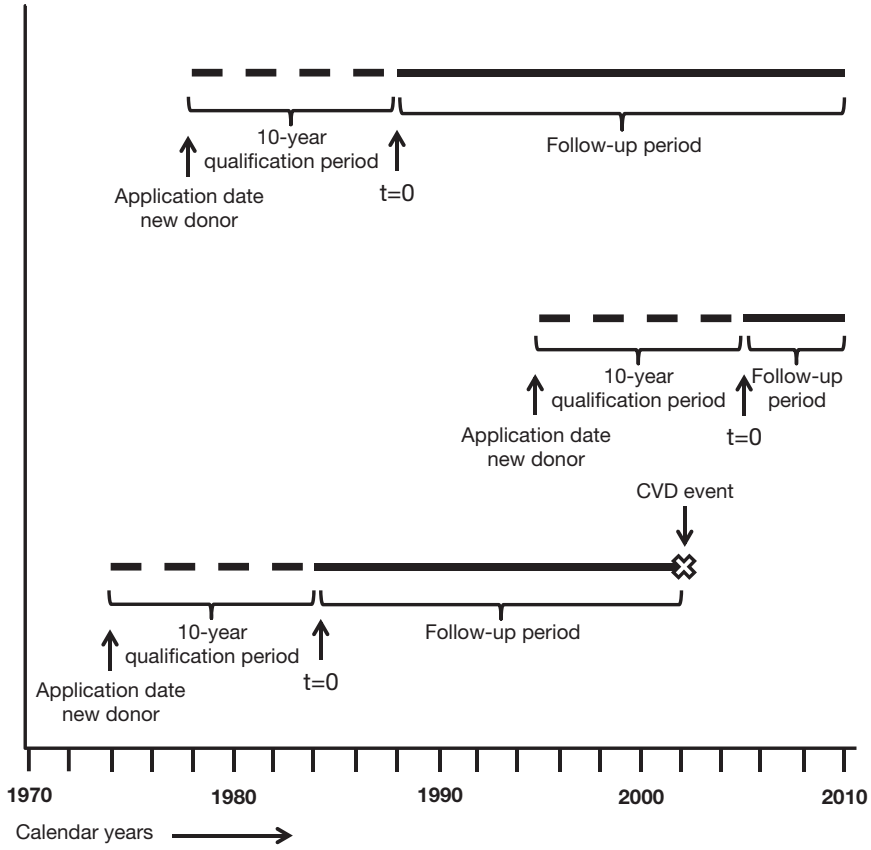


Figure 5.2: Diagram of study design illustrating the 10-year qualification period. Although the qualification period is fixed at 10 years for each donor, the calendar period differs between donors. Donors starting to donate a long time ago will consequently have a longer follow-up period (unless they've become a CVD case). Follow-up was completed until a CVD event occurred or until 31st December 2010. The number of whole-blood donations within the 10-year qualification period is used to create sex-specific donation tertiles.

Mortality The primary outcome in the mortality analysis was cardiovascular death (ICD-9: codes 3900 - 4599; ICD-10: codes I000 - I999) registered as the primary cause of death. This is the underlying cause of death that is primarily responsible for the onset of death. Secondary outcome was cardiovascular death (ICD-9: codes 3900 - 4599; ICD-10: codes I000 - I999) registered as either the primary or secondary cause of death, the latter being causes of death that have contributed to the onset or process of death. Of note, one donor could have both a primary and secondary cardiovascular cause of death, e.g. primary stroke, secondary atherosclerosis.

Statistical analysis

Starting date was calculated from new donor visit or application date, whichever came first. For each donor, duration of career was calculated as time since starting date in months. The number of donations during the 10-year qualification period was used to categorize donors into high-, medium-, or low frequency donors [Figure 5.2]. As men are allowed to donate more often (5 times a year) than women (3 times a year), the cut-offs were based on sex-specific tertiles of number of donations (15 and 22 in men, 12 and 16 in women). All analyses were performed separately for men and women. From year 11 onwards, considered as t=0, donors were followed-up until death or censored at December 31st, 2010.

Hb, blood pressure, and BMI were calculated as individual means throughout the 10-year qualification period and the follow-up period over all donations, including attempts that resulted in a deferral. Donor characteristics were expressed as medians with interquartile ranges, as they appeared to be non-parametrically distributed.

Cumulative survival of primary and secondary cardiovascular cause of death was visualized with Kaplan-Meier curves. A log-rank test was performed to test for differences in survival between the three sex-specific donation tertile groups.

Multivariable Cox regression analyses, with time since 11 donation years (t=0) as person-time, were performed to adjust for the available potential confounding variables one by one. We have considered age (at start of the donor career), Hb, SBP, DBP, BMI, and blood type as potential confounders. Donation tertiles were treated as a categorical variable with the first tertile as reference group. Data preparation and analysis were performed in SPSS 20.0.0.2 for Windows.

Results

Morbidity

From a total of 17,084,348 person-months, the median follow-up time was 108 (interquartile range: 63) months. High-frequency donors had a shorter follow-up time [Table 5.1]. High-frequency donors had more donations during the follow-up period than medium- and low-frequency donors, indicating that they had continued their donation intensity from the 10-year qualification period [Table 5.1]. In women, high-frequency donors were older at the

Table 5.1: Characteristics reported as median (IQR) or N (%) and cardiovascular morbidity across donation tertiles

	Sex-specific donation tertiles of whole-blood					
Characteristics	*Men*			*Women*		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Number of donations	1-14	15-20	21-154	1-11	12-15	16-115
N	30,996	27,247	28,553	26,768	21,108	21,155
Age (y)[a]	35 (14)	36 (16)	36 (16)	29 (14)	32 (16)	36 (17)
Follow-up (months)	118 (60)	112 (60)	104 (69)	112 (65)	108 (64)	90 (73)
Donations follow-up[b]	8 (12)	11 (15)	14 (19)	5 (9)	7 (10)	8 (11)
Blood type						
A^-	2,339 (7.5)	2,351 (8.6)	2,354 (8.2)	2,172 (8.1)	1,956 (9.3)	1,776 (8.4)
A^+	10,332 (33.3)	9,368 (34.4)	8,505 (29.8)	8,538 (31.9)	7,045 (33.4)	6,219 (29.4)
AB^-	278 (0.9)	256 (0.9)	202 (0.7)	253 (0.9)	171 (0.8)	141 (0.7)
AB^+	1,622 (5.2)	617 (2.3)	271 (0.9)	1,174 (4.4)	408 (1.9)	229 (1.1)
B^-	599 (1.9)	541 (2.0)	482 (1.7)	569 (2.1)	451 (2.1)	336 (1.6)
B^+	4,005 (12.9)	1,436 (5.3)	523 (1.8)	3,044 (11.4)	1,166 (5.5)	516 (2.4)
0^-	1,764 (5.7)	2,388 (8.8)	4,541 (15.9)	2,111 (7.9)	2,074 (9.8)	3,444 (16.3)
0^+	10,050 (32.4)	10,281 (37.7)	11,674 (40.9)	8,889 (33.2)	7,828 (37.1)	8,490 (40.1)
Missing	7 (0.0)	9 (0.0)	1 (0.0)	18 (0.1)	9 (0.0)	4 (0.0)
Hb[c] (mmol/l)						
Qualification period	9.6 (0.8)	9.6 (0.7)	9.5 (0.7)	8.5 (0.7)	8.5 (0.6)	8.5 (0.6)
Follow-up period	9.5 (0.7)	9.4 (0.7)	9.4 (0.7)	8.5 (0.7)	8.5 (0.7)	8.6 (0.6)
SBP[c] (mmHg)						
Qualification period	129 (17)	129 (16)	129 (16)	120 (15)	121 (15)	123 (16)
Follow-up period	133 (19)	133 (18)	133 (18)	123 (19)	125 (19)	127 (20)
DBP[c] (mmHg)						
Qualification period	80 (10)	80 (10)	80 (9)	76 (9)	77 (10)	78 (10)
Follow-up period	82 (10)	82 (10)	82 (10)	78 (11)	79 (11)	79 (11)
BMI[c] (kg/m^2)						
Qualification period	25.8 (3.9)	26.0 (3.6)	25.9 (3.8)	24.4 (4.8)	24.7 (4.8)	25.0 (5.0)
Follow-up period	25.8 (3.9)	26.0 (3.6)	25.9 (3.7)	24.4 (4.8)	24.7 (4.8)	25.0 (5.0)

Table 5.1: Characteristics reported as median (IQR) or N (%) and cardiovascular morbidity across donation tertiles (continued)

Characteristics	**Sex-specific donation tertiles of whole-blood**					
	Men			*Women*		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Number of donations	1-14	15-20	21-154	1-11	12-15	16-115
Morbidity						
Primary CV discharge or death	3,500 (11.29)	3,054 (11.21)	2,827 (9.90)	1,761 (6.58)	1,359 (6.44)	1,218 (5.76)
Non-CV death	603 (1.95)	539 (1.98)	527 (1.85)	249 (0.93)	221 (1.05)	233 (1.10)
Primary CV hospital admission or death						
Age-adjusted[a] HRR (95%-CI)	1 (ref.)	1.00 (0.95 - 1.05)	0.99 (0.94 - 1.04)	1 (ref.)	0.93 (0.87 - 1.00)	0.90 (0.84 - 0.97)

Hb, haemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body-mass index; CV, cardiovascular; HRR, Hazard Rate Ratio; CI, confidence interval. Data are reported from valid cases, with 17.6% of donors with missing values in Hb and blood pressure during the qualification period, and 13.4% during follow-up. BMI was missing for 62.6% of donors during qualification period and 64.0% during follow-up. [a] Age at start donation career. [b] Number of whole-blood donations from year 10 onwards. [c] Hb, blood pressure, and BMI are derived from individual means over each visit throughout the qualification period and follow-up period.

start of their donor career than medium- and low-frequency donors; a difference that was not observed among men [Table 5.1]. Women also showed a slightly increased blood pressure with increasing donation tertiles, and this effect carried on during the follow-up period. No trends were observed in Hb or BMI, except for a small increase during the follow-up period in blood pressure for all donation tertiles, irrespective of gender.

A total of 9,381 (10.81%) men and 4,338 (6.28%) women suffered a primary cardiovascular hospital admission or death [Table 5.1]. In men, no significant differences were observed in the Kaplan-Meier curves comparing donation tertiles (p=0.021, Figure 5.3a), whereas high-frequency female donors seemed to have an increased cardiovascular event rate compare to medium- and low-frequency donors [p=0.001, Figure 5.3b]. Adjusted for age, men still not differed in cardiovascular hazard across donation tertiles, with a hazard rate ratio (HRR) of 0.99 (95%-CI: 0.94 to 1.04) [Table 5.1]. However, women showed a reduction in cardiovascular hazard associated with high-frequency blood donation (HRR=0.90, 95%-CI: 0.84 to 0.97) compared to low-frequency donors [Table 5.1].

Mortality

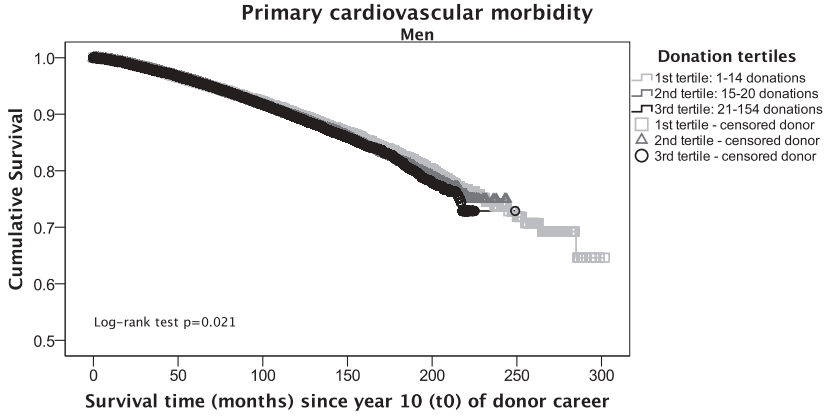
Median follow-up time was 113 (interquartile range: 61) months from a total of 18,128,762 person-months, and was longest in low- and medium frequency donors [Table 5.2]. Medium- and high-frequency donors also donated more often during follow-up, and had more often blood type 0 (especially 0^-) [Table 5.2]. During follow-up, blood pressure increased equally within sex-specific donation tertiles [Table 5.2]. Women in the upper two donation tertiles were older at the start of their donor career and had a slightly higher blood pressure both during the qualification period as well as during follow-up.

Of the 158,919 donors, a total of 3,859 (2.43%) donors were deceased, of whom 875 (0.55%) suffered a primary cardiovascular death, and another 362 (0.23%) suffered a secondary cardiovascular death. Overall death rates and cardiovascular mortality were lower in women than in men [Table 5.2].

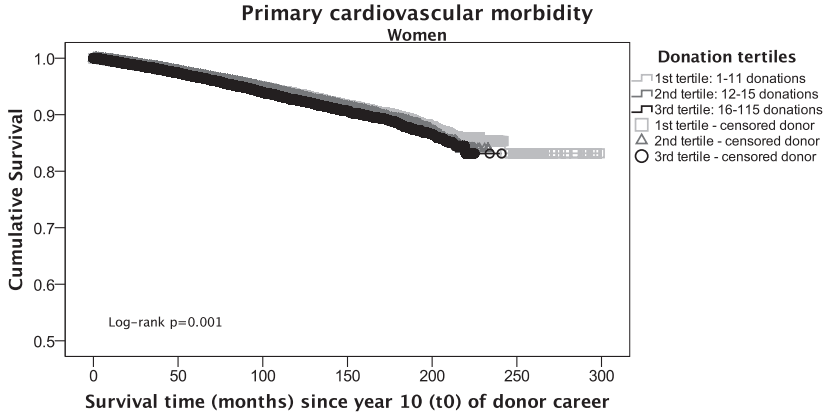
In men, both primary (p=0.526) and primary and secondary cardiovascular survival combined (p=0.234) was comparable across donation tertiles [Figure 5.4a and 5.4b]. The age-adjusted HRR of high-frequency donors comparing the hazard rate of primary cardiovascular death with low-frequency donors was 0.94 (95%-CI: 0.78 to 1.12).

In women, primary cardiovascular survival was also comparable across donation tertiles (p=0.128, Figure 5.5a), whereas primary and secondary cardiovascular survival combined significantly differed (p=0.003, Figure 5.5b) in favour of low- and medium frequency donors. The age-adjusted HRR of primary cardiovascular survival was 0.83 (95%-CI: 0.56 to 1.22) for high-frequency donors compared with low-frequency donors [Table 5.2].

In both men and women, medium-frequency donors had a less-pronounced decrease in primary and secondary cardiovascular death combined than primary cardiovascular death alone, whereas high-frequency donors had an equal hazard rate for primary and secondary cardiovascular death combined compared with low-frequency donors [Table 5.2].



(a) Primary cardiovascular morbidity based on hospital admissions and causes of death in men



(b) Primary cardiovascular morbidity based on hospital admissions and causes of death in women

Figure 5.3: Kaplan-Meier curves of cumulative cardiovascular disease-free survival, separately for sex-specific donation tertiles based on the number of donations in the first 10 years of the donation career. Follow-up starts at year 10 (t=0).

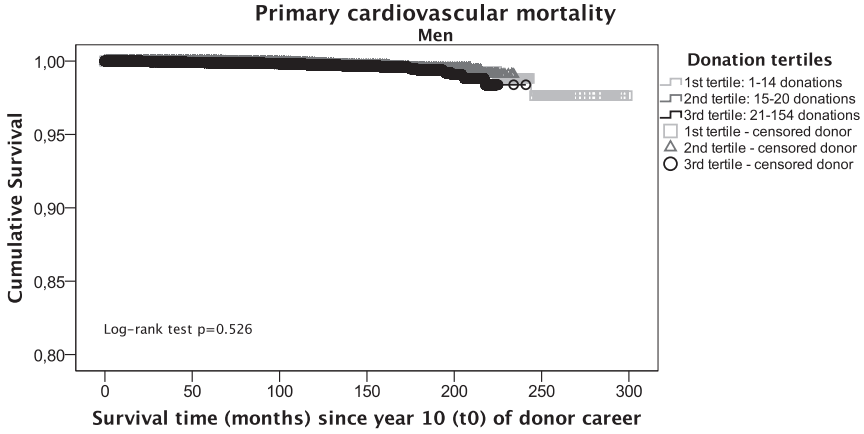
Table 5.2: Characteristics reported as median (IQR) or N (%) and cardiovascular mortality across donation tertiles

Characteristics	**Sex-specific donation tertiles of whole-blood**					
	Men			*Women*		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Number of donations	1-14	15-20	21-154	1-11	12-15	16-115
N	31,430	27,616	29,030	27,428	21,644	21,771
Age (y)[a]	35 (14)	36 (16)	36 (16)	29 (14)	32 (16)	36 (17)
Follow-up (months)	125 (63)	117 (57)	110 (64)	115 (66)	111 (60)	92 (74)
Donations follow-up[b]	8 (12)	11 (15)	14 (19)	6 (9)	7 (10)	8 (11)
Blood type						
A^-	2,368 (7.5)	2,383 (8.6)	2,391 (8.2)	2,228 (8.1)	2,009 (9.3)	1,832 (8.4)
A^+	10,502 (33.4)	9,483 (34.3)	8,669 (29.9)	8,769 (32.0)	7,219 (33.4)	6,436 (29.6)
AB^-	283 (0.9)	259 (0.9)	208 (0.7)	256 (0.9)	177 (0.8)	146 (0.7)
AB^+	1,634 (5.2)	630 (2.3)	276 (1.0)	1,196 (4.4)	424 (2.0)	235 (1.1)
B^-	613 (2.0)	547 (2.0)	495 (1.7)	585 (2.1)	464 (2.1)	349 (1.6)
B^+	4,066 (12.9)	1,452 (5.3)	532 (1.8)	3,122 (11.4)	1,193 (5.5)	531 (2.4)
0^-	1,776 (5.7)	2,422 (8.8)	4,604 (15.9)	2,146 (7.8)	2,114 (9.8)	3,540 (16.3)
0^+	10,181 (32.4)	10,431 (37.8)	11,854 (40.8)	9,109 (33.2)	8,035 (37.1)	8,698 (40.0)
Missing	7 (0.0)	9 (0.0)	1 (0.0)	18 (0.1)	9 (0.0)	4 (0.0)
Hb[c] (mmol/l)						
Qualification period	9.6 (0.8)	9.6 (0.7)	9.5 (0.7)	8.5 (0.7)	8.5 (0.6)	8.5 (0.6)
Follow-up period	9.5 (0.7)	9.5 (0.7)	9.4 (0.7)	8.5 (0.7)	8.5 (0.7)	8.6 (0.6)
Blood pressure[c]						
SBP (mmHg)						
Qualification period	129 (17)	129 (16)	129 (15)	120 (15)	121 (15)	123 (16)
Follow-up period	133 (19)	133 (18)	133 (18)	123 (19)	125 (19)	127 (20)
DBP (mmHg)						
Qualification period	80 (10)	80 (10)	80 (9)	76 (9)	77 (10)	78 (10)
Follow-up period	82 (10)	82 (10)	82 (10)	78 (11)	79 (11)	79 (11)
BMI[c] (kg/m^2)						
Qualification period	25.8 (3.9)	26.0 (3.6)	25.9 (3.8)	24.4 (4.8)	24.7 (4.8)	25.0 (5.0)
Follow-up period	25.8 (3.9)	26.0 (3.6)	25.9 (3.7)	24.4 (4.9)	24.7 (4.8)	25.0 (5.0)

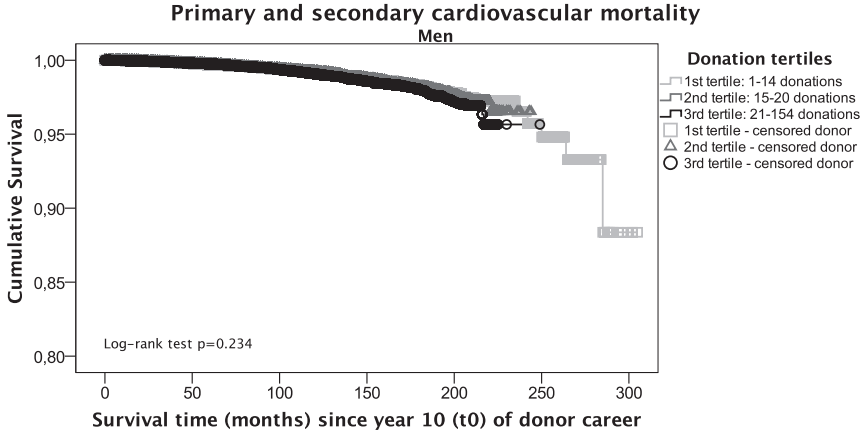
Table 5.2: Characteristics reported as median (IQR) or N (%) and cardiovascular mortality across donation tertiles (continued)

Characteristics	Sex-specific donation tertiles of whole-blood					
	Men			*Women*		
	Low	**Medium**	**High**	**Low**	**Medium**	**High**
Number of donations	1-14	15-20	21-154	1-11	12-15	16-115
Deceased	1059 (3.36)	919 (3.33)	890 (3.07)	364 (1.33)	305 (1.41)	322 (1.48)
Primary CV death	281 (0.89)	217 (0.79)	217 (0.75)	63 (0.23)	48 (0.22)	48 (0.22)
Secondary CV death	104 (0.33)	99 (0.36)	87 (0.30)	25 (0.09)	20 (0.09)	27 (0.12)
Primary or secondary CV death	337 (1.07)	277 (1.00)	276 (0.95)	76 (0.28)	64 (0.30)	69 (0.32)
Primary CV death						
Age-adjusted[a] HRR (95%-CI)	1 (ref.)	0.88 (0.74 - 1.05)	0.94 (0.78 - 1.12)	1 (ref.)	0.82 (0.56 - 1.20)	0.83 (0.56 - 1.22)
Primary or secondary CV death						
Age-adjusted[a] HRR (95%-CI)	1 (ref.)	0.93 (0.79 - 1.09)	0.99 (0.84 - 1.16)	1 (ref.)	0.93 (0.66 - 1.30)	1.01 (0.72 - 1.42)

Hb, haemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body-mass index; CV, cardiovascular; HRR, Hazard Rate Ratio; CI, confidence interval. Data are reported from valid cases, with 17.2% of donors with missing values in Hb and blood pressure during the qualification period, and 13.2% during follow-up. BMI was missing for 62.6% of donors during qualification period and 64.0% during follow-up.Of note: donors could have had a primary and secondary CV cause of death, thus the secondary outcome measurement is less than the sum of primary and secondary CV cases. [a] Age at start donation career. [b] Number of whole-blood donations from year 10 onwards. [c] Hb, blood pressure, and BMI are derived from individual means over each visit throughout the qualification period and follow-up period.

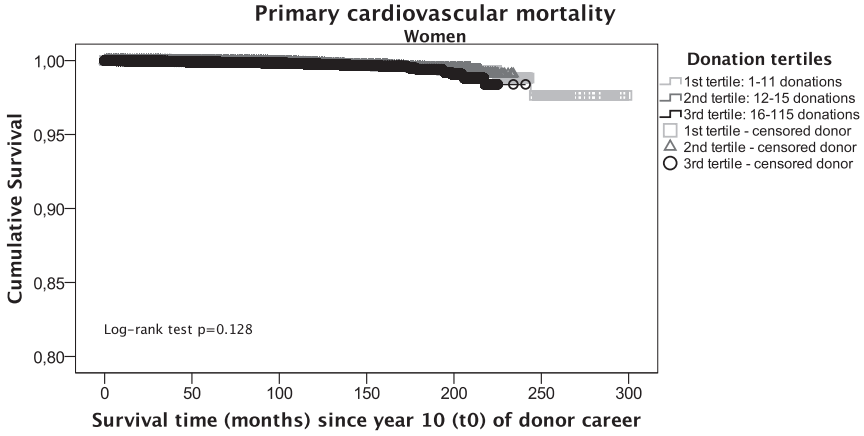


(a) Primary cardiovascular death in men

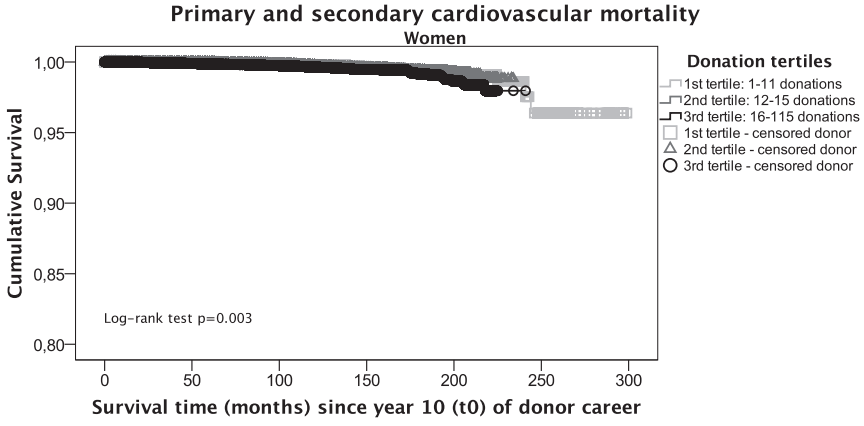


(b) Primary and secondary cardiovascular death combined in men

Figure 5.4: Kaplan-Meier curves of cumulative cardiovascular survival in men, separately for donation tertiles based on the number of donations in the first 10 years of the donation career. Follow-up starts at year 10 (t=0).



(a) Primary cardiovascular death in women



(b) Primary and secondary cardiovascular death combined in women

Figure 5.5: Kaplan-Meier curves of cumulative cardiovascular survival in women, separately for donation tertiles based on the number of donations in the first 10 years of the donation career. Follow-up starts at year 10 (t=0).

Discussion

This study found a protective effect of long-term, frequent whole-blood donation on cardiovascular morbidity and mortality in women from the cohort of all Dutch whole-blood donors, but not in men. Women with a high donation frequency during the first ten years of their donation career had a decrease of 10% in cardiovascular morbidity and 17% in cardiovascular mortality compared to women with a low donation frequency. In men, the respective numbers were 1% and 6%.

Previous studies were often challenged by the healthy donor effect; an effect that would lead to potentially biased results as a consequence of repeatedly applied health criteria to the donor population to commence donorship. We have aimed to minimize such an effect by only including donors that were able to donate for at least 10 years, meaning that they must have had at least one successful whole-blood donation from year 10 onwards and donated at least once in year 8 or 9.

In a simulation study from our own research group, we have previously demonstrated that the use of such a qualification period does decrease the Healthy Donor Effect that normally occurs by comparing high- and low frequency donors based on the lifetime number of donation (unpublished data). When using the lifetime number of donations at the end of follow-up, this will be inevitably associated with survival time since reaching a high number of donations requires more disease-free survival time. The biggest advantage of using a 10-year qualification period, is that the exposure frequency (i.e. number of donations) is determined separately from the follow-up period in which cardiovascular events are measured. The number of blood donations used to compare donors with different exposure levels is therefore no longer per definition related with survival time. However, the simulation study also showed that the HDE-bias is not completely eliminated with a qualification period, especially when the period used is longer and the protective effect of blood donation is small relative to other causal factors of CVD-risk. Nonetheless, this approach still yields better results than the conventional one using lifetime number of donations.

Another advantage of this time-window between exposure and outcome measurement, is the circumvention of the need to use so-called latency periods to avoid reverse causation bias. This means that incipient cardiovascular disease in donors could lead to a lower donation intensity in his/her last years of donorship, and that subsequent cardiovascular death is erroneously associated with a lower donation intensity, frequency, or duration of the donor career. Such an association is less likely in our design, as the exposure was determined at least 10 years before any cardiovascular outcome could have occurred. This study is the first study to have used the qualification period approach to estimate the effect of blood donation frequency on cardiovascular disease.

In a previous study from Meyers et al., a somewhat comparable approach was used with a period of 3 years [47]. However, this period of 3 years was set between 1988 and 1990, and not within the first years or a fixed moment of the donor career. Within these 3 years, donors could qualify themselves as a frequent donor (> 1 unit of whole-blood each year) or a casual donor (1 unit of whole-blood in that 3-year period).

The attempt made by Meyers et al. is very honourable, and their study was actually one of the first that specifically aimed to avoid this bias. The results from Meyers' study indicated a protective effect of frequent blood donation on cardiovascular events (adjusted Odds Ratio of 0.60, 95%-CI: 0.43 to 0.83), a much more pronounced effect than our current study. Of specific interest, the effect of frequent donation was larger in women (adjusted OR=0.25, 95%-CI: 0.09 to 0.73) than in men (adjusted OR=0.67, 95%-CI: 0.47 to 0.94), completely in line with our own findings. Likewise, a recent study on all-cause mortality found that the inverse association between donation rate and mortality was smaller in men (mortality rate ratio, MRR=0.89, 95%-CI: 0.86 to 0.92 per donation/year) than in women (MRR=0.84, 95%-CI: 0.78 - 0.91) [125]. The researchers from this group also made an appreciable effort to eliminate the healthy donor effect by adjusting for a variable that predicted ongoing donation based on the donation rate among donors with a last donation at ages 64.5-65 years who survived at least 2 years thereafter. However, they also could not interpret their results as conclusive evidence of a beneficial health, as they were unsure whether a residual HDE was still present.

This increased protective effect in women seems surprisingly. Until now, men were hypothesised to gain more from regular blood donation as they have higher iron stores [129]. Perhaps women *do* reach a sufficiently low iron level, whereas men *do not*. The fact that Meyers et al. found women to benefit from blood donation was explained by their exclusion of women younger than 50 years at baseline, meaning that virtually all donors were post-menopausal and theoretically able to benefit from iron loss. In contrast, our female donors were all quite young at baseline, still in their early 30s, thus in their early 40s at the start of follow-up. Galesloot et al. also observed that hepcidin and the hepcidin/ferritin ratio were associated with presence of atherosclerotic plaque in postmenopausal women but not in men from the general population [130]. With respect to cardiovascular disease, known risk factors contribute differently to disease risk in men and women, in large part modified by differences in hormonal status affecting lipid profiles, body fat distribution, insulin resistance, endothelial function, and coagulation and fibrinolysis [10–12, 131]. Reducing oxidative stress through blood donation-induced iron depletion could therefore yield increased protective effects in women to counteract the damaging effects of menopause.

A drawback of our design is that our donors might not have had a sufficient follow-up time to investigate cardiovascular mortality; our donors were quite young when they started donating, being in their mid-30s. Requiring a 10-year cardiovascular survival to be included in the study implicates that donors were generally young. As a result, our donors were in their mid-40s when follow-up started, and with a maximum follow-up time of approximately 25 years, our donors were only yet around mid-60s at the end of follow-up. Thus, it is highly likely that our donors were still too young to die of cardiovascular disease; mean age of cardiovascular death was 77 years in men and 84 in women in the general Dutch population in 2012 [7]. Indeed, our results do indicate that age at start of the donation career was an important contributing factor. In the unadjusted analyses in women, the effect estimates pointed towards a slight increased risk of medium- and high-frequency donors (data not

shown), who were also older at the start of their donation careers. When adjusted for age, the effect estimates reversed to a slight protective effect, implying that the age difference was part of the explanation of the initially increased risk in female medium- and high-frequency donors. From a biologic perspective, it should be emphasised that including such young donors at the start of their donation career is important to study true protective effects of blood donation, as it should have ample opportunity to slow down the disease process.

The strongest support for an insufficient follow-up time in our mortality study to demonstrate protective effects, are the results of the morbidity study. Because these occur earlier in life, the power increases to detect differences between the donor groups as more case-patients are observed. Indeed, even with half the incidence of that in men, (borderline) significant effects were observed in women. Of interest though, the size of the effect estimates of blood donation on mortality were larger than on morbidity. The difference between the two cardiovascular endpoints lies in an earlier detection and thus stage of the disease. After being admitted to a hospital for cardiovascular disease, other factors come in play that determine the course and progress of the disease until eventually death occurs, such as treatment. A large contributing factor for the differences between morbidity and mortality thus lies within the therapy that a donor received after having survived the initial cardiovascular incident. Without the availability of treatment data, and without being able to exclude an association with donation frequency in the first ten years, it can be argued that the effect size of our morbidity study is a more accurate measure of the actual effect size of blood donation as therapeutic effects are less expected to have influenced morbidity data.

The current study was short in data on known potential confounding factors such as BMI, blood type, smoking behaviour, dietary iron intake/supplementation, and blood pressure to completely adjust for it or study their contribution to the relation of blood donation and cardiovascular mortality. Assuming that high-frequency donors represent a healthier subdomain of the donor population, with less donors smoking, not accounting for such confounding factors would mean an overestimation of the protective effect of high-frequency blood donation in the current study: a residual healthy donor effect. To investigate the likeliness of such a healthy (self)-selection, we have examined the association between donation frequency and smoking behaviour, medication use (for lipids, hypertension, and glucose), physical activity, and prevalence of metabolic syndrome (MetS) among donors from the CARDON-study (N=633, age $\geq$ 45 years) [74] who have had remained active for at least 10 years and whose measurements were conducted after the 10-year qualification period. In both men (n=315) and women (n=134), the tertiles based on the number of donations in the first 10 years of their donation careers were not statistically significant associated with a higher proportion of never-smokers, ranging from 31% in the lowest tertile to 37% in the highest tertile. Medication use was higher in the second and third donation tertiles in women, even statistically significant for lipid-lowering drugs. No differences were observed among men. Physical activity was lowest in the first donation tertile and highest in the second donation tertile in both sexes. Prevalence of MetS was highest in the third donation tertile, even after adjustment for age at start donation career, BMI, hs-CRP,

smoking, having ever been a plasma donor, having a family history of CVS, physical activity, and postmenopausal status, and this difference persisted upon adjustment for ferritin and hepcidin as a measurement of iron stores and distribution, respectively. These associations indicate that donors with a high donation frequency in the first ten years of their donation career actually have a less favourable cardiovascular risk profile, which therefore unlikely explains the lower morbidity and mortality rate in high-frequency female donors. Of note, our simulation study has also shown that the healthy donor effect increases with increasing causal effects of iron stores (and thus blood donation) on cardiovascular risk. Although we do not know the true causal contribution of iron to cardiovascular risk, given the inconsistencies between the studies conducted so far should indicate that it is unlikely to be a tremendous causal factor. Taken together, our results might not have been that much biased by a residual healthy donor effect, although we cannot rule out completely that (female) donors who donated more often during their first 10 years of their donation career were already more healthy to begin with.

In light of previous studies that were embedded within the donor population, our results provide new insights. No protective effect of frequent blood donation on cardiovascular incidence has been reported since the study from Meyers and co-workers [46, 47, 52].

With the 10-year qualification period decreasing but not completely eliminating the healthy donor effect-bias, this study showed promising reductions in cardiovascular morbidity and mortality associated with high-frequency blood donation during the first 10 years of the donor career, only in women. We conclude that long-term, high frequency blood donation might reduce cardiovascular disease in women, and encourage future research into sex-specific effects of blood donation on cardiovascular disease.

Chapter 6

General Discussion

The aim of this thesis was to test whether lowering iron stores by means of frequent blood donation prevents cardiovascular disease, thereby taking into account the methodologically challenging healthy donor effect. The main finding of this thesis is that blood donation could indeed protect against cardiovascular disease in women, but not through metabolic improvements.

By tapping each aspect of developing cardiovascular disease, moving from early cardiometabolic risk factors, to subclinical atherosclerosis, to cardiovascular illness, and eventually to cardiovascular death, this thesis has attempted to open the black box of blood donation and cardiovascular disease. How do the findings piece together and what is the state of affairs?

6.1 Account and Accountability

In Chapters 2, 3, 4, and 5 a flow of the research was presented that was conducted within diverse study populations, as to give substance to the preventative hypothesis of blood donation. We started off exploring the immediate and short-term effects of blood donation, all the way across the continuum of biochemical pathways, towards subclinical atherosclerosis and clear manifestations of cardiovascular morbidity and mortality.

Cardiometabolic Risk

The availability of data and cost constraints for additional measurements have driven us first to conduct a study among 120 first-time donors at baseline, aged ≥45 years, who were invited by postal mail to participate in the so-called CARdiovascular risk and DONation (CARDON)-study [Section 2.1]. After a median period of 20 months, all of them were re-invited to participate in follow-up measurements for insulin resistance (HOMA2-IR) and other cardiometabolic risk factors. Within that follow-up period, they showed a range of 0-9 blood donations, with a median number of 3. We observed that iron stores were greatly reduced by the donations, but insulin resistance increased instead of decreased as was hypothesized, especially in women. The observed increase in insulin resistance was best explained by ageing.

The following study was intended to employ the concept of blood donation in greater detail by looking at the intensity of donation: a combination of donation career in years and donation frequency per year [Section 2.2]. To this extent, the CARDON-study was expanded to a random sample of 422 male and 211 female active whole-blood donors, ≥45 years of age, for a cross-sectional study. Lipids, glucose and iron parameters were measured after overnight fasting and presence of metabolic syndrome (MetS) was determined. Again, iron levels were lower in high-intensity donors. MetS appeared to be present in one-quarter of the donors. We made contrast on the donation exposure by categorization of donation intensity into low, medium, or high based on sex-specific tertiles of donor career and donation frequency per year. Unexpectedly, the low and high part of the donation spectrum did not yield consistent differences in MetS prevalence. Results suggested a higher prevalence of MetS in high intensity donors; an effect most pronounced in non-obese women. It should be mentioned, though, that iron parameters did not follow the hypothesis either as they appeared not to be involved in any of these associations.

Subclinical Atherosclerosis

The next step was to explore the more stringent parts of the disease spectrum with the inclusion of subclinical atherosclerosis [Section 3.1]. From the large-scale population-based Nijmegen Biomedical Study (NBS) we were able to match 1491 NBS-2 participants (aged 50-70 year) to Sanquin's blood bank registry based on sex, date of birth, and last name. Of these, 272 persons were linked to the blood bank registry, and NBS data on non-invasive

measurements of atherosclerosis (NIMA), blood variables, and a self-administered questionnaire were added. NIMA measurements consisted of carotid IMT of the common carotid artery, ankle-brachial index (ABI), and pulse-wave velocity (PWV). Across the sex-specific tertile groups of lifetime number of donations containing approximately 90 donors each, ferritin and hepcidin were lower in high-frequency donors compared to low-frequency donors. Donors in the third sex-specific donation tertile had on average a 0.3% lower IMT values, a 2.1% higher PWV value, and a 1.5% higher ABI compared to donors from the first tertile. With such small differences and no consistent trend across donation groups, it could not be concluded that blood donation has a beneficial benefit on the extent of subclinical atherosclerosis. In interpreting the findings, however, a few things became clear, especially that time since last donation in the first tertile was 9.7 yr, whereas in the third tertile it was 1.4 yr. Therefore, we performed a post-hoc analysis among currently active donors (at least one donation in the last two years), which confirmed the null-results found in the entire study population.

Qualification Period

For the next study, we wanted to investigate cardiovascular morbidity and mortality using 'big data' from Sanquin Blood Bank of all Dutch whole-blood donors ever. Although we had already reduced the 'Healthy Donor Effect' phenomenon by not comparing the donation population with the general population, we were still facing selection and confounding issues as defined by Femke Atsma and co-workers. Because overt cardiovascular disease more likely occurs outside the donation career, comparing high-frequency donors with low-frequency donors should not become a comparison of active with stopped donors. Besides, blood donation was assumed to require a longer period of commencement to prove its preventive capabilities on such longterm outcomes. As a possible solution to both issues, we wanted to adopt a qualification period of ten years: whole-blood donors should have remained active for at least ten years. Adherence to such a qualification period of donation was the central prerequisite. After that, donors were categorised into donation tertiles according to the total number of whole-blood donations during this 10-year qualification period.

Healthy Donor Effect

Whether such a qualification period would successfully eliminate the HDE-bias but still left room for detecting a preventive effect of blood donation, was investigated with a simulation study [Section 4.1]. The endeavours of a cohort of 1,000,000 persons were simulated, with a maximum follow-up of 300 months during which donating lowered iron stores. Cardiovascular risk increased based on iron stores and lifestyle, representing all other causal risk factors of CVD. Transition probabilities from being a non-donor to an active donor, and from active to stopped donor, as well as the probability to donate were either completely random or dependent on CVD-risk: the selection processes inducing HDE. Both simulation scenarios (with a random or healthy donor selection) were analysed with the conventional lifetime

number of donations approach and a 1, 5, 10, 15 and 20-year qualification period approach. Cox proportional hazards modeling estimated the crude and lifestyle-adjusted hazard rate ratio (HRRs) for high- vs. low-frequency donors, a categorisation that was either based on the number of donations during the qualification period or the lifetime number of donations. Using the lifetime number of donations severely overestimated the protective effect of blood donation.

Even when no causal effect of iron stores was simulated and donors were randomly selected, high-frequency donors appeared to have a reduced cardiovascular hazard rate (HRR=0.67); a time-effect that is entirely attributed to the association between the lifetime number of donations and disease-free survival time. More mimicking reality with the scenario of a small causal effect of iron stores and a healthy donor selection, the HRR of the qualification periods decreased with increasing qualification periods to converge to the conventional lifetime approach (0.16), with HRR=0.83 for a 1-year qualification period and HRR=0.17 for a 20-year qualification period. The magnitude of the HDE-bias increased with longer qualification periods and a larger causal effect of iron levels on CVD-risk. Adjusting for lifestyle attenuated the HRRs only in the scenario of a healthy donor selection, and especially when the causal effect of iron levels was relatively small and longer qualification periods were applied. Using qualification periods does decrease the magnitude of the HDE-bias, but does not completely eliminate this type of bias.

The lessons learnt from the simulation were that the qualification period is better than the conventional approach of lifetime number of donations in eliminating the HDE-bias, and on the pivotal influence of the duration of the qualification period affecting the magnitude of the HDE-bias. Another important lesson was the relatively large contribution of other causal CVD factors that acted as confounders due to the healthy donor selection processes, mainly in the situation where the true preventive effect of lower iron levels is small. This emphasises the need to measure all other causal CVD factors.

Cardiovascular Outcomes

With this knowledge, we carefully continued investigating cardiovascular morbidity and mortality, applying the new and stringent design considerations regarding to the healthy donor effect [Section 5.1]. A qualification period of ten year was used to include donors and follow them up on cardiovascular morbidity (investigated in 155,827 donors) and mortality (investigated in 158,919 donors) using hospital discharge diagnoses and death certificates from Statistics Netherlands. The median age at start of donor career appeared to be 34 yr (IQR: 16). For this young population, the median follow-up time until cardiovascular hospital admission, death, or end-of study, was 108 (interquartile range: 63) months from a total of 17,084,348 person-months. A total of 9,381 (10.81%) men and 4,338 (6.28%) women had a primary cardiovascular hospital admission or death. Compared with low-frequency blood donation and adjusted for age at start of the donation career, high-frequency blood donation was associated with a reduced cardiovascular morbidity in women (hazard rate

ratio, HRR=0.90, 95%-CI: 0.84 to 0.97), but not in men (HRR=0.99, 95%-CI: 0.94 to 1.04). The population in which cardiovascular mortality was studied had a slightly larger follow-up time, with a median of 113 (interquartile range: 61) months from a total 18,128,762 person-months. Primary cardiovascular mortality was confirmed in 876 (0.55%) donors. The relation between donation frequency during the first ten years of the donation career and primary cardiovascular mortality was more pronounced than was observed with cardiovascular morbidity (women: HRR=0.83, 95%-CI: 0.56 to 1.22, men: HRR=0.94, 95%-CI: 0.78 to 1.12). The young age of this population could have resulted in an insufficient follow-up period to detect the required number of cardiovascular fatalities for statistical significance, but the point estimates seem promising.

The only protective effect found was that on cardiovascular morbidity and an encouraging but not (yet) statistical significant effect on mortality; can we now conclude that blood donation is preventive for cardiovascular disease? A critical appraisal of the results learns that the HDE and perhaps residual "confounding" is the actual explanation of the observed (small) protective effects of blood donation. As the simulation study relies on many assumptions of not only the magnitude of the causal effect of iron levels, but also the strength of the healthy donor selection, we must address if and how much the HDE-bias accounts for the results, as well as other sources of influence from choosing specific study designs and -populations.

6.2 Methodological Considerations

The hypothesis that blood donation lowers cardiovascular disease risk mainly relies on the subsequent decrease in oxidative stress due to iron loss. Oxidative stress not only affects glucose metabolism by increasing insulin resistance, but also damages the vascular endothelium directly and enhances the formation of foam cells in the atherosclerotic process. These are all intermediary outcomes of cardiovascular disease. This thesis has not found protective effects of blood donation on such intermediary outcomes, but a (slight) protective effect on cardiovascular morbidity and promising effects on cardiovascular mortality were found in women. How should the discrepancies in these results be explained? Does the choice for specific study populations, in order to minimize a type of bias called the healthy donor effect, have a role in this? Throughout this thesis, this methodologically challenging aspect of performing health research in donors has been addressed. Do the main findings of this thesis now point to a causal effect of blood donation on cardiovascular disease risk, or should they be interpreted as biased results? This section addresses several methodological aspects that need to be considered before arriving at final conclusions.

Study Populations

All of the studies in this thesis were conducted within the donor population at large, i.e. not using information from the general population. Because of all the health criteria that donors must meet in order to become a donor and continue donating, they are generally healthy.

As such, they might not have had that much to gain from blood donation, with already low cardiovascular disease risks not really an *at-risk* group, and probably not that high in body iron stores. Then again, the participants from the CARDON-study were intentionally selected on their slightly increased risk because one of the inclusion criteria was an age of at least 45 years. This to ensure that early deteriorations in cardiovascular risk would be detectable at all. Likewise, the age criteria of the Nijmegen Biomedical Study participants (50 - 70 years) increased the likelihood of detecting subclinical atherosclerosis.

However, it could have occluded a cardiovascular benefit if they had already been exposed too long to the damaging and irreversible effects of iron and other cardiovascular risk factors. If irreversible damage had already occurred, either by the damaging effects of iron-catalysed hydroxyl radicals or other factors, these donors wouldn't have had that much to gain from blood donation. The importance of the age at which someone starts donating seems to be confirmed by the findings of a protective effect in the population that was relatively young (median age 34 years) in which cardiovascular morbidity and mortality was studied, whereas no beneficial effects were found in the CARDON-study on insulin resistance which included first-time donors with a median age of 52. Of note, the age at start of the donation career is unlikely to explain the null-results in either the CARDON-study on metabolic syndrome (median age 36 years) or the Nijmegen Biomedical Study on subclinical atherosclerosis (median age 39 years).

When considering the possible dose-effect relationship between iron and cardiovascular disease, one could imagine that there is a steep curve in the upper range of iron levels, and a much more flat line in the lower range of iron levels. Therefore, when someone has really high iron levels, a decrease of, say 50%, could have tremendous beneficial effects on cardiovascular disease risk, whereas such a decrease in donors with already low iron levels would not have a similar effect on their CVD risk. This seems plausible as many observational studies have found high ferritin levels to be predictive of type 2 diabetes mellitus and cardiovascular disease, but when lowering iron stores in blood donors there is not a directly protective effect [Chapters 2 and 3], rather only a long-term one [Chapter 5].

Since our donors are generally healthy, it can be assumed that they would have iron levels in the low-to-normal-range. This is plausible because chronic subclinical inflammation increases hepcidin expression [24, 132] and ferritin also acts as an acute phase reactant [133], both increasing systemic iron levels in diseased individuals [59]. This iron increase is also often seen in chronic metabolic conditions [21], making it quite difficult to test the iron-hypothesis, as it remains unsure whether iron affects the metabolic alterations or the other way around.

Most of the studies in this thesis showed higher blood pressure and body weight/BMI with increasing donation intensity. If this association is relatively stronger than that between lower iron stores and a reduced cardiometabolic risk, than this could be the explanation of our null-/contra hypothesized results. If metabolically less healthy donors happen to donate more often, then high-intensity donors would have an increased cardiometabolic risk that is probably not to be averted with lowering iron stores through donating blood. In this case, it

would take a lot more time for blood donation to counteract such associations and reveal its protective effect.

Therefore, the duration of the donation career could also be of great importance, and some of the studies in this thesis may lack a sufficient time-window to truly reveal a protective effect. It can be argued that only continuing donating for at least 10 years with an adequate number of whole-blood donations per year would be sufficient to truly lower one's cardiovascular risk. This idea seems to be confirmed by the protective findings of blood donation on cardiovascular morbidity and mortality [Chapter 5]. In some of the other studies in this thesis, however, the number of blood donations and the duration of the donor career were possibly insufficient, not providing enough time to exert beneficial effects of repeated blood donation.

Healthy Donor Effect

One of the largest scientific concerns of previous studies that aimed to investigate the health effects of blood donation, was a type of bias that is referred to as the healthy donor effect (HDE). Although blood donors are a subgroup of the general population, they actually differ in many respects from their source population.

First of all, there are physical entry requirements for blood donors. First-time donors must be between 18 and 65 years old, whereas active blood donors may continue to donate until they are 70 years old. The average age of Dutch whole-blood donors is consequently around 45, whereas this is 40.6 years for the general population in 2012. In light of this thesis' scope, other criteria are more important such as blood pressure (50 - 100 mmHg diastolic and 90 - 180 mmHg systolic), body weight (> 50 kg), and capillary haemoglobin level (7.8 - 11.0 mmol/L for women and 8.4 - 12.0 mmol/L for men before donation).

Second, donors differ in psychological profile and (health) behaviour from the general population. Donors are more often religious, married, higher educated, low-risk takers, and have a higher socio-economic status than non-donors [134, 135]. Behaviour that is accompanied with an increased risk for infectious disease is less common among blood donors. Moreover, donors highly appreciate the medical screening before each donation. As a result, donors constitute a healthier self-selected subdomain of the general population.

All of the above criteria and factors also contribute to differences between low- and high-intensity donors and long- and short-career donors [136]. Someone's health status affects the likelihood of a future donation [53]. These factors would thus correlate well with donation frequency and duration of the donation career, thereby being potentially important confounding factors.

Furthermore, blood donation could affect some of these factors causally. Haemoglobin levels decrease following blood donation due to erythrocyte loss. Blood pressure could also be (temporarily) decreased after donating blood [97]. In addition, haemoglobin and blood pressure are longitudinally related to each other [137]. This makes haemoglobin and blood pressure also intermediary factors at the same time, which means that these factors are

not only causally related to blood donation and cardiovascular disease, but are also direct effects of blood donation. This makes it impossible to simply adjust for these factors, as it would completely over-adjust the effect of blood donation on cardiovascular disease, leading to a null-result.

Atsma and co-workers have laid an important foundation for the subsequent handling of the Healthy Donor Effect in donor research [138]. They have not only identified different types or moments of selection inducing the HDE, they have also provided scientific evidence of the magnitude of the HDE [53]. Comparing high-frequency donors to low-frequency donors within the currently active donor population yielded the smallest deviations from equality [53]. Until now, the recommendation from Atsma and co-workers to embed donor research within the active donor population was leading. However, when studying disease outcomes that also occur outside the active donor career, such as cardiovascular mortality, a need for another approach arises to avoid comparing active donors with stopped donors.

Simulation models

Separating the period in which donation frequency is determined from the period in which the outcome can occur, was hypothesized to reduce the bias as imposed by the Healthy Donor Effect. This can be achieved by the application of a qualification period, which means that only donors who were able to remain an active donor throughout the entire qualification period are selected. Then, the number of donations within this qualification period is used to distinguish high- and low-frequency donors. Using simulation models that mimicked the protective effect of blood donation on CVD risk, the effect of analysing techniques on datasets with and without a healthy donor selection were compared [Section 4.1].

One of the most interesting findings from the simulations was that even without a healthy donor selection (and even without a protective effect of blood donation on CVD risk), there appeared to be a Healthy Donor Effect bias when comparing the hazard rate of CVD events between high-frequency and low-frequency donors based on the total lifetime number of donations. This is a result of high-frequency donors having a higher survival probability, regardless of beneficial effects of blood donation or pre-donation selection on health criteria. In fact, high- and low-frequency donors are already selected on the cardiovascular outcome, as the lifetime number of donations is directly related with survival-time. This phenomenon is quite comparable to the relation between the number of birthdays and someone's age.

The most important result from this simulation study is the near-complete elimination of the HDE-bias when a very short qualification period is applied, but this increases to that observed in the conventional lifetime number of donations approach when the qualification period increases to 20 years. The second most important finding is that a larger contribution of ferritin to CVD risk, i.e. a larger protective effect of blood donation, increases the magnitude of the HDE-bias. They seem to positively feedback one another.

Moreover, the balance between ferritin and other causal risk factors determined how the magnitude of the HDE-bias changed over different qualification periods. A smaller effect of ferritin on CVD-risk increases the importance of other causal risk factors, especially when longer qualification periods are applied. These other causal CVD-factors then operate as confounders, even though they are not directly associated with donation frequency.

These findings combined, the HDE-bias is smallest when the qualification period is short (one year) and the causal effect of ferritin is small. Naturally, this approach is not plausible in situations where exposure has a lagged or long-term effect on disease occurrence, such as assumed in blood donation and cardiovascular disease. Longer qualification periods are then required. The simulation study emphasises the importance of adjusting for any other causal factor of CVD when such a 10-year qualification period is applied, especially if the true protective effect of blood donation is small relative to these causal factors of CVD.

Disease Mechanisms

Besides other or unknown pathways, this thesis has solely focused on one of two openly hypothesized branches (iron and viscosity) of mechanistic pathways from blood donation to a reduced CVD risk: iron [Figure 1.3]. The other hypothesised pathway, viscosity, has not yet received that much attention in relation to blood donation in the scientific literature [40, 41]. However, it has deserved a profound place in the clinical literature, and is a widely recognised risk factor for rupture of vulnerable atherosclerotic plaques [139]. Viscosity translates best as the thickness or stickiness of blood. Increased viscosity means an increased number of cells in the blood and a subsequent increased frictional resistance of the moving blood with the stationary vascular wall. This friction or shear stress is an important biomechanical risk factor for a vulnerable plaque to erode and rupture [139]. Moreover, during diastole, more blood cells will reside along the endothelium, especially in places of low shear stress, enhancing the aggregation of erythrocytes and thrombocytes and creation of a thromboembolism [42].

These aspects were not specifically addressed in this thesis. Since the studies in this thesis did not confirm the pathways of metabolic improvements or subclinical atherosclerosis, studies pursuing the effects of blood donation on viscosity are encouraged to study alternative pathways. Such studies could use the same methodology, as viscosity gradually decreases with volumes drawn. The number of blood donations could therefore be a good determinant to investigate the effects of blood donation on viscosity and atherosclerosis. Because of this resemblance, it seems unlikely that studies hypothesising an effect of blood donation on viscosity and atherosclerosis would yield entirely different results. However, not all effects of viscosity on cardiovascular disease act through atherosclerosis, thus leaving alternative mechanistic pathways as an explanation of the inconsistencies in the studies in this thesis. Additionally, studies that would simultaneously investigate effects of blood donation on viscosity and atherosclerosis could provide new insights into possible overlapping mechanisms, such as lipid profile changes.

6.3 Further Issues

We now have learned that blood donation likely does not immediately improve cardiometabolic risk factors, but its preventive effects on cardiovascular disease cannot be excluded either. This congruency could point to the pathway of atherosclerosis, perhaps via viscosity, although no effects were found on a subclinical level in this thesis. Also, other pathways might be involved. What does this thesis holds for future science, and which issues should be borne in mind?

The Struggle of Subject Matter and Methodology

Throughout this thesis, the healthy donor effect has led to the search for alternative methodologies. Meanwhile, the biologic concept of blood donation, lowering iron stores, and decreasing cardiovascular disease has required other methodological approaches. The idea that a specific contrast should be present in the donation spectrum, directly imposes the possibility of the HDE. In search for the optimal contrast between donor groups, using the lifetime number of donations, the donation frequency per year, and the duration of the donor career, supports the biologic concept of blood donation. At the same time, increasing the contrast in the donation spectrum increases the possibility of HDE-bias.

When beginning from the methodological perspective, the simulation study showed that a qualification period of 1 year had the least biased results in terms of the HDE. However, the effect of such a short period of blood donation on reducing cardiovascular disease seems hardly plausible from a biologic point of view, which actually suggested a 10-year qualification period. Future research should be aware of the importance of starting age, the duration of the donor career, and the way the true causal effect of blood donation directly determines the magnitude of the HDE-bias.

Does Gender Matter?

The studies described in this thesis used tertile scores of the number of donations to determine high- and low-frequency donors. These tertiles were consistently calculated separately in men and women, and all but one study also presented the results separately for men and women. Was there a need to do so? One reason was the different donation trajectories that men and women have; men are allowed to donate more often than women, and they also differ in psychological terms from each other in blood donation behaviour. For example, no-show behaviour, stress responses, coping with adverse reactions of blood donations lead to different donation careers in men and women.

Another reason is the effect of blood donation on iron homeostasis; men tend to normally build up their iron levels over the years, whereas women stay at a relatively low but stable level until menopause as a result of menstrual blood loss and pregnancies. The exact effect of blood donation on iron homeostasis could therefore very well differ between men and women. The last reason is that, combined with the above mentioned factors, men and

women have a different causal risk model for CVD that is still not entirely understood [10]. Thus, it seemed appropriate to treat men and women separately in the analyses.

The results of the individual studies confirm the need for separate analyses of men and women, and are in accordance with gender differences that have also been observed in other studies [47, 102, 104, 130]. However, there are many inconsistencies in the direction of the observed differences. In some instances, only men seem to benefit from blood donation [44]. This seems plausible as they are the 'naturally' high-risk group in terms of iron levels [129]. However, other studies found a beneficial effect that is larger in (postmenopausal) women [47, 49].

The results from the cross-sectional CARDON-study among active donors also point to probable differences in the association of iron parameters and metabolic syndrome prevalence [Section 2.2]. In men, high ferritin levels were mainly associated with metabolic syndrome prevalence, whereas hepcidin was more closely related with metabolic syndrome cases in women. Interestingly, others have argued that the combination of hepcidin and ferritin, as represented by the hepcidin-to-ferritin ratio, is more meaningful in determining subjects at high risk for cardiovascular disease [140]. The biggest clue to different effects in men and women in this thesis was the study on cardiovascular morbidity and mortality, with only an effect in women. As it yet remains undetermined, continuing the separate analysis of men and women seems appropriate.

Beyond Cardiovascular Disease

How should the overall result of this thesis be interpreted in light of other, comparable studies? In Table 6.1, an overview of all studies that have been conducted in healthy subjects who were or could have been blood donors, is provided. It covers a total of 17 studies among which only 3 trials, and includes 4 studies from this thesis. A total of 7 concluded a beneficial effect of blood donation.

So far, all of the previous observational studies that *did* find an effect of blood donation, are highly likely explained by the Healthy Donor Effect, except for the retrospective cohort study by Meyers et al., also using a somewhat similar approach with a 3-year index period in which donation frequency is determined. They concluded that frequent blood donation is associated with a reduced cardiovascular risk, especially in women. This is quite comparable to the study in this thesis on cardiovascular morbidity and mortality with a 10-year qualification period, in which a small protective effect was observed in women but not in men [Chapter 5]. Strikingly, the only three known trials to date, all concluded beneficial effects, albeit at short-term.

Table 6.1: Overview of studies in healthy subjects meeting eligibility criteria for blood donation

Author	Year	Study design	N (% men)	Age	Donation contrast[a]	Outcome	Follow-up
Observational studies confirming beneficial effects of blood donation							
Salonen [45]	1998	Prospective	2,682 (100)	42-60	1	AMI	9 y
Meyers [44]	1997	Prospective	3,855 (53)	40-103	1	CVD events	5-8 y
Meyers [47]	2002	Retrospective	2,260 (70)	58 ±6	3	CVD events	10 y
Fernández-Real [48]	2005	Cross-sectional	21 (100)	53.8 ±11.3	3	IS	0.5-5 y
Zheng [49]	2005	Cross-sectional	82 (60)	59 ±1	3	FMD	2 y
Zheng [50]	2007	Cross-sectional	42 (60)	57 IQR: 4-7	3	FMD, IS	2 y
Peffer	2015	Retrospective	155,827 (56)	34 IQR: 16	3	CVD events	9 y
Observational studies rejecting beneficial effects of blood donation							
Ascherio [46]	2001	Prospective	38,244 (100)	46-81	1	CHD+MI	4 y
van Hoydonck [141]	2004	Cross-sectional	80 (100)	50 ±7.2	1	Vascular integrity[b]	≥ 4y[c]
Engberink [51]	2008	Cross-sectional	365 (77)	59.1 ±5.4	3	cIMT	32 y
Peffer [105]	2013	Cross-sectional	269 (57)	60.6 ±5.7	3	cIMT, ABI, PWV	14.4 y
Germain [52]	2013	Retrospective	63,246 (60)	38.6	2	CHD events	10 y
Peffer [74]	2015	Cross-sectional	633 (67)	55.1 ±5.8	3	MetS	17.7 y
Borai [73]	2015	Prospective	42/10 (100)	36±7	3,4	IR	3 w/4 m
Peffer	2015	Prospective	112 (46)	52.2 ±5.0	3,4	IR	1.5-2 y
Ullum [125]	2015	Retrospective	1,182,495 (56)	18-64	3	all-cause mortality	8 y
Controlled trials confirming beneficial effects of blood donation							
Salonen [142]	1995	Cross-over	14 (100)	30-63	4	oxLDL	30 w
van Jaarsveld [143]	2002	Non-randomized	23 (100)	37.6 ±12.4	4	oxLDL, NO	6-8 w
Houschyar [97]	2012	Single-blinded RCT	64 (58)	59 ±8.4	5	SBP (no effect IS)	6 w

[a] 1=donors vs. non-donors; 2=active vs. ex-donors; 3=high- vs. low-frequency donors; 4=before-after; 5=donor vs. control. [b] Measured as OxLDL, s-ICAM-1, sVCAM-1, an vWF-antigen. [c] Derived from inclusion criterion of ≥ 20 donations. ABI, ankle-brachial index; (A)MI, (acute) myocardial infarction; CHD, coronary heart disease; cIMT, carotid intima media thickness; CVD, cardiovascular disease; FMD, flow-mediated dilation; IS/IR, insulin sensitivity/-resistance; IQR, interquartile range; MetS, metabolic syndrome; NO, nitric oxide; oxLDL, oxidized LDL-cholesterol; PWV, pulse wave velocity.

New horizons in blood donor research?

In the context of healthy individuals and as a preventive measure, the studies described in this thesis provide sufficient cause for future research into blood donation and cardiovascular disease. The most crucial role is to confirm the underlying pathway through which blood donation operates, including the effects on iron metabolism and gender differences herein. This thesis points to the likeliness of mechanisms other than the metabolic one (i.e. insulin resistance and metabolic syndrome). Studies not only aiming at atherosclerosis, but also viscosity, should be further pursued. Moreover, gender differences in the benefits from blood donation deserve more attention.

Apart from cardiovascular disease, blood donation could also have positive effects on other important illnesses with a high burden of disease. For example, iron has been implied in the development of neurological disorders and cancer. During ageing, iron accumulates in the brain and stimulates the aggregation of amyloid plaques as found in Alzheimer's disease [144]. Blood donation has also been hypothesised to reduce cancer risk by lowering iron [145]. The oxidative stress induced by high iron levels could also result in DNA damage, and thus increase cancer risk [146]. Furthermore, malignant cells require more iron for their increased cell division rate, suggesting that iron facilitates tumour growth [147]. This means that lowering oxidative stress through blood donation could reduce cancer risk, as found in two previous studies [148, 149], although another study found no effects on colorectal cancer incidence and mortality [150].

However, the repeated removal of erythrocytes and immunoglobulins increases cell proliferation, and increased cell proliferation could actually increase the risk of malignancies [151]. In a large linkage study in the Danish and Swedish donor population, plasma donation was surprisingly associated with an increased risk for non-Hodgkin lymphoma [123]. Such findings require further investigation in blood donors.

6.4 Conclusion

This thesis indicates a protective effect of blood donation on cardiovascular disease in women, but improving the metabolic condition through iron depletion is unlikely to be involved. Rather, other pathways such as a reduced viscosity or the combination with atherosclerotic plaque formation play a role. In future studies aiming at these mechanistic pathways, gender differences in the effect of blood donation on cardiovascular disease as well as on altering iron metabolism need to be explored. The health of blood donors should be adequately monitored as iron stores are depleted and long-term effects, both positive and negative, on other physiological compartments are not excluded. Any such effects of blood donation should be known in order to maintain a blood supply that solely relies on healthy, voluntary, non-remunerated (or uncompensated) donors.

Appendix A

Appendix to Section 2.2

Additional analyses on metabolic syndrome prevalence in non-obese and obese donors.

Table A1: Prevalences and Prevalence Ratios (95%-CIs) of metabolic syndrome (MetS) in non-obese and obese donors

Sex	Obesity	Donation Intensity	MetS N(%)	Prevalence Ratio (95%-CI)				
				Unadjusted	Adjusted[a]			
						+MCV	+Ferritin	+Ferritin +Hepcidin
Men	Non-obese (< 30 kg/m^2)	Low N=117	18 (15.4)	Ref.	Ref.	Ref.	Ref.	Ref.
		Medium N=134	18 (13.4)	0.87 (0.48-1.60)	0.93 (0.50-1.73)	0.96 (0.51-1.77)	1.05 (0.56-1.98)	1.06 (0.56-1.99)
		High N=110	22 (20.0)	1.30 (0.74-2.29)	1.30 (0.72-2.33)	1.37 (0.75-2.49)	1.57 (0.86-2.90)	1.58 (0.86-2.92)
	Obese (≥ 30 kg/m^2)	Low N=13	8 (61.5)	Ref.	Ref.	Ref.	Ref.	Ref.
		Medium N=25	16 (64.0)	1.04 (0.62-1.75)	1.18 (0.71-1.97)	1.18 (0.71-1.97)	1.11 (0.68-1.82)	1.07 (0.68-1.68)
		High N=23	18 (78.3)	1.27 (0.79-2.06)	1.24 (0.79-1.93)	1.24 (0.79-1.93)	1.15 (0.75-1.75)	1.10 (0.74-1.63)
Women	Non-obese (< 30 kg/m^2)	Low N=61	3 (4.9)	Ref.	Ref.	Ref.	Ref.	Ref.
		Medium N=60	12 (20.0)	4.07 (1.21-13.69)	3.43 (1.01-11.64)	3.11 (0.93-10.40)	3.49 (1.02-11.97)	3.42[b] (0.99-11.85)
		High N=55	12 (21.8)	4.44 (1.32-14.90)	3.84 (1.10-13.40)	3.78 (1.11-12.94)	3.94 (1.10-14.12)	3.89[b] (1.06-14.23)
	Obese (≥ 30 kg/m^2)	Low N=7	4 (57.1)	Ref.	Ref.	Ref.	Ref.	Ref.
		Medium N=16	7 (43.8)	0.77 (0.33-1.79)	0.58 (0.27-1.26)	0.71 (0.34-1.95)	0.62 (0.30-1.27)	0.62 (0.29-1.32)
		High N=12	7 (58.3)	1.02 (0.46-2.27)	0.64 (0.29-1.44)	0.93 (0.35-2.43)	0.77 (0.35-1.69)	0.80 (0.32-2.00)

[a] Adjusted model includes: age (continuous), hs-CRP (continuous), smoking (categorical, 3 levels), having ever been a plasma donor (dichotomous), having a family history of CVD (dichotomous), and postmenopausal status (dichotomous). [b] Model did not converge. MCV, mean corpuscular volume; hs-CRP, high-sensitivity C-reactive protein; CVD, cardiovascular disease.

Appendix B

Appendix to Section 4.1

Syntaxes used to create simulated datasets with six different scenarios:

1. no causal effect, random donor selection [Listing B.1]
2. no causal effect, healthy donor selection [Listing B.2]
3. small or large causal effect, random donor selection [Listing B.3]
4. small or large causal effect, healthy donor selection [Listing B.4]

Listing B.1: No causal effect, random donor selection

```
1
2  SET RNG = MT MTINDEX = 592004.
3
4  NEW FILE.
5  INPUT PROGRAM.
6  LOOP #x=1 TO 1000000.
7  COMPUTE Ferritin= RV.NORMAL(100,20).
8  COMPUTE Ferritin_fake= RV.NORMAL(100,20).
9  COMPUTE random1=RV.UNIFORM(0, 1).
10 COMPUTE random2=RV.UNIFORM (0,1).
11 COMPUTE randomCVD=RV.UNIFORM(0,1).
12 COMPUTE randomCVD2=RV.NORMAL(100,20).
13 COMPUTE lifestyle=RV.UNIFORM(0,100).
14 COMPUTE Donation=0.
15 COMPUTE CVD_event=0.
16 COMPUTE persontime=0.
17 COMPUTE stop=0.
18 COMPUTE Donor=0.
19 COMPUTE Number_Donations=0.
20 IF (random1 <0.01) Donor=1.
21 IF (Donor=1) start=0.
22 COMPUTE baselineCVD=RV.UNIFORM(0,1).
23 COMPUTE CVD= baselineCVD + 0.001*Ferritin_fake.
24 IF (Ferritin <10) Ferritin=10.
25 END CASE.
26 END LOOP.
27 END FILE.
28 END INPUT PROGRAM.
29 EXECUTE.
30 /*********************************.
31
32 LOOP #month=1 TO 300.
33 COMPUTE Donation=0.
34 COMPUTE random2=RV.UNIFORM(0,1).
35 COMPUTE randomCVD=RV.UNIFORM(0,1).
36 COMPUTE randomCVD2=RV.UNIFORM(0,1).
37 /*Creating new donors, with moment of becoming a new donor saved in start.
38 DO IF (Donor=0 AND (random2 > 0.995)).
39 COMPUTE Donor=1.
40 COMPUTE start=#month.
41 END IF.
42 /*Creating stopped donors, with moment of ending donor career saved in stop.
43 DO IF (Donor=1 AND (random2 <0.005)).
44 COMPUTE Donor=2.
45 COMPUTE stop=#month.
46 END IF.
47 /*Making sure that non-donors really remain clean, blanco, new donors and not already increase iron levels or have CVD events.
48 DO IF Donor NE 0.
49 IF (Donation=0 AND Donor=1 AND (random2)>0.5) Donation=1.
50 COMPUTE Number_Donations=Number_Donations + Donation.
51 IF (Donation=1) Last_Donation=#month.
52 IF (Donation=1) Ferritin=Ferritin - 10.
53 /*Creating a fake ferritin variable that mimics the pattern of ferritin but is independent of blood donation.
54 IF (randomCVD2>0.775) Ferritin_fake=Ferritin_fake - 10.
55 /*Small causal effect ferritin_fake: 0.0005*Ferritin_fake and 0.0002*lifestyle.
56 COMPUTE CVD= CVD + (0.0005*Ferritin_fake) + (0.0002*lifestyle).
57 IF ((randomCVD<0.00001) OR (randomCVD<0.0001 AND CVD >1) OR (randomCVD<0.001 AND CVD >2.5) OR (randomCVD<0
       .01 AND CVD>5)) CVD_event=1.
58 IF (CVD_event=1) event_time=#month.
59 COMPUTE Ferritin=Ferritin+1.
```

```
60 IF (Ferritin<10) Ferritin=10.
61 COMPUTE Ferritin_fake=Ferritin_fake +1.
62 IF (Ferritin_fake<10) Ferritin_fake=10.
63 /*Saving total Number Donations and donor status at the end of each qualification period.
64 DO IF #month = start=12.
65 COMPUTE Donations_qual_1y=Number_Donations.
66 COMPUTE donor_qual_1y=Donor.
67 END IF.
68 DO IF #month = start=60.
69 COMPUTE Donations_qual_5y=Number_Donations.
70 COMPUTE donor_qual_5y=Donor.
71 END IF.
72 DO IF #month = start=120.
73 COMPUTE Donations_qual_10y=Number_Donations.
74 COMPUTE donor_qual_10y=Donor.
75 END IF.
76 DO IF #month = start=180.
77 COMPUTE Donations_qual_15y=Number_Donations.
78 COMPUTE donor_qual_15y=Donor.
79 END IF.
80 END IF.
81 END LOOP IF CVD_event=1.
82 EXECUTE.
```

Listing B.2: No causal effect, healthy donor selection

```
SET RNG = MT MTINDEX = 592004.

NEW FILE.
INPUT PROGRAM.
LOOP #x=1 TO 1000000.
COMPUTE Ferritin= RV.NORMAL(100,20).
COMPUTE Ferritin_fake=RV.NORMAL(100,20).
COMPUTE random1=RV.UNIFORM(0, 1).
COMPUTE random2=RV.UNIFORM (0,1).
COMPUTE randomCVD=RV.UNIFORM(0,1).
COMPUTE randomCVD2=RV.NORMAL(0,1).
COMPUTE lifestyle=RV.UNIFORM(0,100).
COMPUTE Donation=0.
COMPUTE CVD_event=0.
COMPUTE persontime=0.
COMPUTE stop=0.
COMPUTE Donor=0.
COMPUTE Number_Donations=0.
IF (random1 <0.01) Donor=1.
IF (Donor=1) start=0.
IF (Donor=1) baselineCVD = RV.UNIFORM(0,0.5).
IF (Donor=0) baselineCVD=RV.UNIFORM(0,1).
COMPUTE CVD= baselineCVD + 0.001*Ferritin_fake.
IF (Ferritin <10) Ferritin=10.
END CASE.
END LOOP.
END FILE.
END INPUT PROGRAM.
EXECUTE.
*********************************.

LOOP #month=1 TO 300.
COMPUTE Donation=0.
COMPUTE random2=RV.UNIFORM (0,1).
COMPUTE randomCVD=RV.UNIFORM(0,1).
COMPUTE randomCVD2=RV.UNIFORM(0,1).
/*Recalculating baselineCVD for non-donors, to ensure sufficient number of new donors.
IF (Donor=0) baselineCVD= RV.UNIFORM(0,1).
/*Creating new donors, with moment of becoming a new donor saved in start.
DO IF (Donor=0 AND (random2 > 0.99) AND baselineCVD<0.5).
COMPUTE Donor=1.
COMPUTE start=#month.
END IF.
/*Creating stopped donors, with moment of ending donor career saved in stop.
DO IF Donor=1 AND ((random2<0.0005 AND CVD>1) OR (random2<0.005 AND CVD>2.5) OR (random2<0.05 AND CVD>5)).
COMPUTE Donor=2.
COMPUTE stop=#month.
END IF.
/*Making sure that non-donors really remain clean, blanco, new donors and not already increase iron levels or have CVD events.
DO IF Donor NE 0.
IF (Donation=0 AND Donor=1 AND ((random2>0.5 AND CVD <2.5) OR (random2>0.75 AND CVD <5))) Donation=1.
COMPUTE Number_Donations=Number_Donations + Donation.
IF (Donation=1) Last_Donation=#month.
IF (Donation=1) Ferritin=Ferritin - 10.
/*Creating a fake ferritin variable that mimics the pattern of ferritin but is independent of blood donation.
IF (randomCVD2>0.775) Ferritin_fake=Ferritin_fake - 10.
/*Small causal effect ferritin_fake: 0.0005*Ferritin and 0.0002*lifestyle.
COMPUTE CVD= CVD + (0.0005*Ferritin_fake) + (0.0002*lifestyle).
```

```
60 IF ((randomCVD<0.00001) OR (randomCVD<0.0001 AND CVD >1) OR (randomCVD<0.001 AND CVD >2.5) OR (randomCVD<0
       .01 AND CVD>5)) CVD_event=1.
61 IF (CVD_event=1) event_time=#month.
62 COMPUTE Ferritin=Ferritin+1.
63 IF (Ferritin<10) Ferritin=10.
64 COMPUTE Ferritin_fake=Ferritin_fake +1.
65 IF (Ferritin_fake<10) Ferritin_fake=10.
66 /*Saving total Number Donations and donor status at the end of each qualification period.
67 DO IF #month = start=12.
68 COMPUTE Donations_qual_1y=Number_Donations.
69 COMPUTE donor_qual_1y=Donor.
70 END IF.
71 DO IF #month = start=60.
72 COMPUTE Donations_qual_5y=Number_Donations.
73 COMPUTE donor_qual_5y=Donor.
74 END IF.
75 DO IF #month = start=120.
76 COMPUTE Donations_qual_10y=Number_Donations.
77 COMPUTE donor_qual_10y=Donor.
78 END IF.
79 DO IF #month = start=180.
80 COMPUTE Donations_qual_15y=Number_Donations.
81 COMPUTE donor_qual_15y=Donor.
82 END IF.
83 END IF.
84 END LOOP IF CVD_event=1.
85 EXECUTE.
```

Listing B.3: Small or large causal effect, random donor selection

```
SET RNG=MT MTINDEX=592004.

NEW FILE.
INPUT PROGRAM.
LOOP #x=1 TO 1000000.
COMPUTE Ferritin= RV.NORMAL(100,20).
COMPUTE random1=RV.UNIFORM(0, 1).
COMPUTE random2=RV.UNIFORM(0,1).
COMPUTE randomCVD=RV.UNIFORM(0,1).
COMPUTE lifestyle=RV.UNIFORM(0,100).
COMPUTE Donation=0.
COMPUTE CVD_event=0.
COMPUTE persontime=0.
COMPUTE stop=0.
COMPUTE Donor=0.
COMPUTE Number_Donations=0.
IF (random1 <0.01) Donor=1.
IF (Donor=1) start=0.
COMPUTE baselineCVD=RV.UNIFORM(0,1).
/*Small causal effect:.
/*COMPUTE CVD= baselineCVD + 0.001*Ferritin.
/*Large causal effect:.
COMPUTE CVD= baselineCVD + 0.005*Ferritin.
IF (Ferritin <10) Ferritin=10.
END CASE.
END LOOP.
END FILE.
END INPUT PROGRAM.
EXECUTE.
**********.

LOOP #month=1 TO 300.
COMPUTE Donation=0.
COMPUTE random2=RV.UNIFORM (0,1).
COMPUTE randomCVD=RV.UNIFORM(0,1).
/*Creating new donors, with moment of becoming a new donor saved in start.
DO IF (Donor=0 AND (random2 > 0.995)).
COMPUTE Donor=1.
COMPUTE start=#month.
END IF.
/*Creating stopped donors, with moment of ending donor career saved in stop.
DO IF (Donor=1 AND (random2 <0.005)).
COMPUTE Donor=2.
COMPUTE stop=#month.
END IF.
/*Making sure that non-donors really remain clean, blanco, new donors and not already increase iron levels or have CVD events.
DO IF Donor NE 0.
IF (Donation=0 AND Donor=1 AND (random2)>0.5) Donation=1.
COMPUTE Number_Donations=Number_Donations + Donation.
IF (Donation=1) Last_Donation=#month.
IF (Donation=1) Ferritin=Ferritin - 10.
/*Small causal effect ferritin: 0.0005*Ferritin and 0.0002*lifestyle.
/*COMPUTE CVD= CVD + (0.0005*Ferritin) + (0.0002*lifestyle).
/*Large causal effect ferritin: 0.001*Ferritin and 0.0001*lifestyle.
COMPUTE CVD= CVD + (0.001*Ferritin) + (0.0001*lifestyle).
IF ((randomCVD<0.00001) OR (randomCVD<0.0001 AND CVD >1) OR (randomCVD<0.001 AND CVD >2.5) OR (randomCVD<0
        .01 AND CVD>5)) CVD_event=1.
IF (CVD_event=1) event_time=#month.
COMPUTE Ferritin=Ferritin+1.
IF (Ferritin<10) Ferritin=10.
```

```
/*Saving total Number Donations and donor status at the end of each qualification period.
DO IF #month = start=12.
COMPUTE Donations_qual_1y=Number_Donations.
COMPUTE donor_qual_1y=Donor.
END IF.
DO IF #month = start=60.
COMPUTE Donations_qual_5y=Number_Donations.
COMPUTE donor_qual_5y=Donor.
END IF.
DO IF #month = start=120.
COMPUTE Donations_qual_10y=Number_Donations.
COMPUTE donor_qual_10y=Donor.
END IF.
DO IF #month = start=180.
COMPUTE Donations_qual_15y=Number_Donations.
COMPUTE donor_qual_15y=Donor.
END IF.
END IF.
END LOOP IF CVD_event=1.
EXECUTE.
```

Listing B.4: Small or large causal effect, healthy donor selection

```
SET RNG=MT MTINDEX=592004.

NEW FILE.
INPUT PROGRAM.
LOOP #x=1 TO 1000000.
COMPUTE Ferritin= RV.NORMAL(100,20).
COMPUTE random1=RV.UNIFORM(0, 1).
COMPUTE random2=RV.UNIFORM(0,1).
COMPUTE randomCVD=RV.UNIFORM(0,1).
COMPUTE lifestyle=RV.UNIFORM(0,100).
COMPUTE Donation=0.
COMPUTE CVD_event=0.
COMPUTE persontime=0.
COMPUTE stop=0.
COMPUTE Donor=0.
COMPUTE Number_Donations=0.
IF (random1 <0.01) Donor=1.
IF (Donor=1) start=0.
IF (Donor=1) baselineCVD=RV.UNIFORM(0,0.5).
IF (Donor=0) baselineCVD=RV.UNIFORM(0,1).
/*Small causal effect:.
COMPUTE CVD= baselineCVD + 0.001*Ferritin.
/*Large causal effect:.
/*COMPUTE CVD= baselineCVD + 0.005*Ferritin.
IF (Ferritin <10) Ferritin=10.
END CASE.
END LOOP.
END FILE.
END INPUT PROGRAM.
EXECUTE.
*********************************.

LOOP #month=1 TO 300.
COMPUTE Donation=0.
COMPUTE random2=RV.UNIFORM (0,1).
COMPUTE randomCVD=RV.UNIFORM(0,1).
/*Recalculating baselineCVD for non-donors, to ensure sufficient number of new donors.
IF (Donor=0) baselineCVD= RV.UNIFORM(0,1).
/*Creating new donors, with moment of becoming a new donor saved in start.
DO IF (Donor=0 AND (random2 > 0.99) AND baselineCVD<0.5).
COMPUTE Donor=1.
COMPUTE start=#month.
END IF.
/*Creating stopped donors, with moment of ending donor career saved in stop.
DO IF Donor=1 AND ((random2<0.0005 AND CVD>1) OR (random2<0.005 AND CVD>2.5) OR (random2<0.05 AND CVD>5)).
COMPUTE Donor=2.
COMPUTE stop=#month.
END IF.
/*Making sure that non-donors really remain clean, blanco, new donors and not already increase iron levels or have CVD events.
DO IF Donor NE 0.
IF (Donation=0 AND Donor=1 AND ((random2>0.5 AND CVD <2.5) OR (random2>0.75 AND CVD <5))) Donation=1.
COMPUTE Number_Donations=Number_Donations + Donation.
IF (Donation=1) Last_Donation=#month.
IF (Donation=1) Ferritin=Ferritin - 10.
/*Small causal effect ferritin: 0.0005*Ferritin and 0.0002*lifestyle.
COMPUTE CVD= CVD + (0.0005*Ferritin) + (0.0002*lifestyle).
/*Large causal effect ferritin: 0.001*Ferritin and 0.0001*lifestyle.
/*COMPUTE CVD= CVD + (0.001*Ferritin) + (0.0001*lifestyle).
IF ((randomCVD<0.00001) OR (randomCVD<0.0001 AND CVD >1) OR (randomCVD<0.001 AND CVD >2.5) OR (randomCVD<0
        .01 AND CVD>5)) CVD_event=1.
```

```
IF (CVD_event=1) event_time=#month.
COMPUTE Ferritin=Ferritin+1.
IF (Ferritin<10) Ferritin=10.
*Saving total Number Donations and donor status at the end of each qualification period.
DO IF #month = start=12.
COMPUTE Donations_qual_1y=Number_Donations.
COMPUTE donor_qual_1y=Donor.
END IF.
DO IF #month = start=60.
COMPUTE Donations_qual_5y=Number_Donations.
COMPUTE donor_qual_5y=Donor.
END IF.
DO IF #month = start=120.
COMPUTE Donations_qual_10y=Number_Donations.
COMPUTE donor_qual_10y=Donor.
END IF.
DO IF #month = start=180.
COMPUTE Donations_qual_15y=Number_Donations.
COMPUTE donor_qual_15y=Donor.
END IF.
END IF.
END LOOP IF CVD_event=1.
EXECUTE.
```

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Summary

Cardiovascular disease was the leading cause of death in The Netherlands until 2007, and remains a significant contributor to the total burden of disease. Iron has been implied as a cause of cardiovascular disease in 1981 by Sullivan. This hypothesis was originally used to explain the sex difference in the occurrence of heart disease. In men, cardiovascular disease has an earlier onset with a higher incidence rate than in women. Within women, there is a gross distinction between pre- and postmenopausal disease occurrence. The incidence of cardiovascular disease is much lower in premenopausal women, but rapidly increases after menopause to the rate that is observed in men. Meanwhile, iron stores remain at a low level due to menstrual blood loss and pregnancies, but steadily increases during menopause, whereas men have increasing iron levels throughout adulthood. Therefore, lowering iron stores was proposed to have a protective impact on cardiovascular disease occurrence.

The mechanism behind remains to be elucidated, but the catalyzing role of iron in the formation of reactive oxygen species, especially hydroxyl radicals, is central to the hypothesis. The damaging effects of such reactive oxygen species on DNA, LDL-cholesterol, and the vascular endothelium could promote the process of atherosclerosis, and indirectly through metabolic alterations leading to insulin resistance and ultimately cardiovascular disease.

Blood donation has been an effective measure to deplete iron stores. Each whole-blood donation (500 ml) contains approximately 200 - 250 mg of iron; a significant amount compared to the average total body iron of 3000 - 4000 mg. To compensate this loss, blood donors must increase their dietary iron absorption, as there is no active iron secretory mechanism that can be reduced to maintain iron levels.

One of the methodological challenges when relating blood donation to reduced cardiovascular disease is to prevent biased results due to the healthy donor effect. Because donors are repeatedly selected on health criteria prior to each blood donation, blood donors are generally healthier than non-donors, but also health differences between high- and low-frequency donors occur due to these selections. As a result, (high-frequency) blood donation appears to reduce cardiovascular disease whereas it is actually a result of a healthy donor selection. The studies in this thesis were therefore confined to blood donors. Furthermore, the application of a qualification period to participate in the individual studies to further exclude the healthy donor effect was explored in a simulation study.

The effects of blood donation on cardiometabolic risk were studied in two different donor populations from the CARdiovascular risk and DONation (CARDON) study. The CARDON-study measured cardiometabolic risk factors in first-time whole-blood donors and currently active whole-blood donors aged 45 years and older. Insulin resistance did not improve in the cohort of first-time blood donors that was followed-up for 1.5 - 2 years [Section 2.1]. More long-term effects of blood donation were studied in a cross sectional sample of currently active blood donors. Metabolic Syndrome prevalence was not lower in high-intensity donors compared with low-intensity donors [Section 2.2]. Thus, the CARDON-study did not provide evidence for a protective effect of blood donation on cardiometabolic risk.

The protective effects of blood donation on vascular integrity was examined in a sample of (ex-) blood donors that participated in the Nijmegen Biomedical Study, aged 50 - 70 years. Measurements of subclinical atherosclerosis were compared between high- and low-frequent donors. No differences were observed in either ankle-bracial index, carotid intima-media thickness, or pulse-wave velocity between the two donor groups [Section 3.1].

While studying long-term effects of blood donation such as cardiovascular disease, the HDE-bias was raised to the matter. Because these cardiovascular events more frequently occur outside the active donation career, comparing high-frequency donors to low-frequency donors must not become a comparison of active with stopped donors. Furthermore, blood donation would require a longer time to truly prevent such long-term outcomes. As a possible solution to both issues, a qualification period was adopted. Donors that had remained active in donating for at least ten years were categorized into low- and high-frequency donors, after which they were followed-up for cardiovascular events.

In a simulation study, the effectiveness of applying a 10-year qualification period to eliminate the HDE-bias yet leaving a detectable causal effect of blood donation, was investigated [Section 4.1]. Compared to the conventional lifetime number of donations approach, the qualification period approach yielded less biased results, especially when short (1-year) periods were applied and the causal effect of iron or thus blood donation was relatively small.

Continuing the study on cardiovascular outcomes as registered by Statistics Netherlands and Dutch Hospital Data, high-frequency blood donation during the first 10 years of the donation career was protective against cardiovascular morbidity, but only in women. Because the median age at start of the donation career was only 34 years, this study probably lacked the power to statistically confirm effects on cardiovascular mortality [Section 5.1], although the size and direction of the point estimate were encouraging.

The studies in this thesis did not find any protective effects of blood donation on cardiometabolic risk or subclinical atherosclerosis. However, cardiovascular morbidity and mortality seemed to be reduced among donors with a high donation frequency in the first ten years of their donation career, but only in women. This discrepancy in results could either point to biased results or a true causal effect of blood donation on cardiovascular disease not acting through cardiometabolic risk reduction or subclinical atherosclerosis. The simulation study partly supports the validity of the result on cardiovascular morbidity and mortality, but a residual HDE-bias cannot be entirely excluded. The discrepancy in the results of this thesis could also indicate the presence of an alternative causal pathway through which blood donation lowers cardiovascular disease.

The effects of long-term blood donation are largely unknown, other than the effects on depleting iron stores and reducing whole blood viscosity. The latter reflects the thickness and stickiness of blood, which is reduced by whole blood donation through the removal of cells and plasma substances such as proteins. Viscosity is a known risk factor for the erosion and rupture of vulnerable atherosclerotic plaques and the formation of thromboembolisms. This thesis has not addressed this alternative causal pathway of viscosity. While the number of blood donations would gradually reduce whole blood viscosity, the effects on atherosclerosis as presented in this thesis would largely resemble that of a hypothetical study on viscosity and atherosclerosis. However, the thromboembolism formation affected by viscosity requires new research, and could be the alternative causal pathway explaining the discrepancy of this thesis' results.

This thesis points to a protective effect of whole-blood donation on cardiovascular disease in women, but not in men. However, it seems unlikely that such a protective effect is the result of metabolic improvements and maybe even not through the direct effects on atherosclerosis, but rather is exerted indirectly through a reduced blood viscosity, or even through a yet unidentified mechanism. Research into the mechanism behind this, including gender differences herein, as well as other possible long-term effects of blood donation, are next steps.

Samenvatting

Tot 2007 vormden hart- en vaatziekten de belangrijkste doodsoorzaak in Nederland en zijn zij nog altijd verantwoordelijk voor een groot deel van de totale ziektelast. In 1981 opperde Sullivan dat ijzer een oorzakelijke rol zou spelen bij hart- en vaatziekten. Deze hypothese was oorspronkelijk bedoeld om verschillen tussen mannen en vrouwen te verklaren in het optreden van hart- en vaatziekten. Bij mannen treedt hart- en vaatziekten niet alleen eerder op maar ook sneller vergeleken met vrouwen. Bij vrouwen is er verder een onderscheid te maken tussen pre- en postmenopauzale vrouwen. De hart- en vaatziekten-incidentie is aanzienlijk lager bij premenopauzale vrouwen dan bij postmenopauzale vrouwen, maar stijgt snel vanaf de menopauze tot aan het niveau dat men bij mannen ziet. Tegelijkertijd is het ijzerniveau laag vanwege menstrueel bloedverlies en zwangerschappen en stijgt vanaf de menopauzeleeftijd, terwijl mannen hun volwassen leven lang ijzer blijven stapelen. Daarom zou het verlagen van de ijzervoorraad wel eens beschermende effecten kunnen hebben op het optreden van hart- en vaatziekten.

Het onderliggende mechanisme is nog altijd onduidelijk, maar de catalyserende rol van ijzer bij de vorming van reactieve zuurstofdeeltjes, waarvan met name de hydroxylradicalen belangrijk zijn, speelt een centrale rol in de hypothese. De schadelijke effecten van deze zuurstofdeeltjes op het DNA, het LDL-cholesterol en het vasculaire endotheel zou het ontstaan van atherosclerose bevorderen, maar ook indirect via metabole veranderingen en het ontstaan van insuline resistentie kunnen zij uiteindelijk tot hart- en vaatziekten leiden.

Bloeddonatie is een effectieve methode om de ijzervoorraad op te gebruiken. Elke volbloeddonatie (500 ml) bevat ongeveer 200 - 250 mg ijzer; een significante hoeveelheid ten opzichte van de totale ijzervoorraad van zo'n 3000 - 4000 mg. Om dit verlies te compenseren, zullen bloeddonors de hoeveelheid ijzer die zij uit de voeding opnemen moeten verhogen, omdat er geen actief uitscheidingsmechanisme bestaat voor ijzer dat omlaag zou kunnen om zo toch het ijzerniveau te behouden.

Wanneer men bloeddonatie wil relateren aan een verlaagd cardiovasculair risico, blijkt het een grote uitdaging te zijn om vertekende resultaten te voorkomen als gevolg van het "healthy donor effect". Omdat bloeddonors herhaaldelijk worden geselecteerd op gezondheidscriteria voorafgaand aan elke bloeddonatie, zullen bloeddonors doorgaans gezonder zijn dan niet-donors. Maar ook tussen veel- en weinig-gevers kunnen gezondheidsverschillen ontstaan door deze selecties. De studies in dit proefschrift zijn daarom beperkt tot bloeddonors alleen. Daarnaast is middels een simulatiestudie onderzocht of het toepassen van een kwalificatieperiode om in de afzonderlijke studies deel te nemen, het healthy donor effect verder kan uitsluiten.

De effecten van bloeddonatie op het cardiometabole risico zijn onderzocht in twee verschillende populaties van de CARdiovasculaire risico en DONatie (CARDON)-studie. In de CARDON-studie zijn cardiometabole risicofactoren bestudeerd in de groep van eerste-donatie volbloeddonors en huidige actieve volbloeddonors in de leeftijd van 45 jaar en ouder. Insulineresistentie bleek niet te verbeteren in een cohort van eerste-donatie donors die 1,5 - 2 jaar gevolgd werden [Sectie 2.1]. De meer lange-termijn effecten van bloeddonatie werden bekeken in een transversaal onderzoek bij een steekproef van huidige actieve volbloeddonors. De prevalentie van metabool syndroom was bij hoog-intensieve donors niet lager vergeleken met laag-intensieve donors [Sectie 2.2]. Kortom, de CARDON-studie leverde geen bewijs voor een beschermend effect van bloeddonatie op het cardiometabole risico.

De beschermende effecten van bloeddonatie op de vasculaire integriteit werden onderzocht in een steekproef van (ex-) bloeddonors die meegedaan hadden aan de Nijmegen Biomedische Studie in de leeftijd van 50 - 70 jaar. Metingen van subklinische atherosclerose werden vergeleken tussen veel- en weinig-gevers. Er werden geen verschillen gevonden in de enkel-arm-index, de intima-media dikte van de arteria carotis of de polsgolfsnelheid tussen de beide donorgroepen [Sectie 3.1].

Terwijl het effect van bloeddonatie op langetermijneffecten werd onderzocht, zoals hart- en vaatziekten, werd het HDE opnieuw aan de orde gebracht. Omdat zulke cardiovasculaire incidenten vaak buiten de actieve donatiecarrière optreden, moest de vergelijking tussen veel- en weiniggevers niet een vergelijking worden tussen actieve- en gestopte donors.

Daarnaast zou bloeddonatie een langere tijd nodig hebben om daadwerkelijk zulke langetermijnuitkomsten te kunnen voorkomen. Als mogelijke oplossing voor beide problemen werd een kwalificatieperiode aangenomen. Donors die minstens tien jaar actief donor waren gebleven werden gecategoriseerd in veel- en weiniggevers, waarna zij gevolgd werden voor cardiovasculaire incidenten.

In een simulatiestudie werd de effectiviteit onderzocht van een 10-jaars kwalificatieperiode om de vertekening door HDE te verwijderen terwijl er wel een detecteerbaar causaal effect van bloeddonatie overblijft [Sectie 4.1]. Vergeleken met de conventionele benadering waarbij het levenslange aantal donaties werd gebruikt, leidde de kwalificatieperiode benadering tot minder vertekende resultaten, vooral indien er korte (1-jaars) periodes werden toegepast en het causale effect van ijzer oftewel bloeddonatie relatief klein was.

Verdergaand met het onderzoek naar cardiovascular uitkomsten zoals geregistreerd door het Centraal Bureau voor de Statistiek en de Landelijke Medische Registratie, bleken donors die tijdens de eerste tien jaar van hun donatiecarrière vaak hadden gedoneerd beschermd te zijn tegen cardiovasculaire morbiditeit, maar dit was enkel bij vrouwen het geval [Sectie 5.1]. Omdat de mediane leeftijd bij aanvang van de donatiecarriére slechts 34 jaar was, had deze onderzoekspopulatie waarschijnlijk onvoldoende power om de effecten op cardiovasculaire sterfte statistisch te kunnen bevestigen [Sectie 5.1], hoewel de grootte en richting van de puntschatting bemoedigend waren.

De onderzoeken in dit proefschrift vonden geen beschermende effecten van bloeddonatie op het cardiometabole risico of de vasculaire integriteit. Daarentegen leken de cardiovasculaire morbiditeit en mortaliteit verlaagd te zijn bij donors met een hoge donatiefrequentie in de eerste tien jaar van hun donatiecarrière, maar dit was enkel bij vrouwen het geval. Deze discrepantie in de resultaten zouden kunnen wijzen op vertekende resultaten, óf op een waarlijk causaal effect van bloeddonatie op hartvaatziekte dat niet verloopt via het verlagen van het cardiometabole risico of subklinische atherosclerose. De simulatiestudie onderbouwt deels de validiteit van de bevindingen op cardiovasculaire morbiditeit en mortaliteit, maar een residuele vertekening door het HDE kan niet geheel worden uitgesloten. De discrepantie in de resultaten van dit proefschrift kunnen evenwel duiden op de aanwezigheid van een alternatief causaal mechanisme waarmee bloeddonatie een beschermend effect heeft op hart- en vaatziekten.

De langetermijneffecten van bloeddonatie zijn grotendeels onbekend, behalve dan de effecten op het opgebruiken van de ijzervoorraad en het verlagen van de volbloedviscositeit. Laatstgenoemde geeft de dikte en plakkerigheid van het bloed weer, welke verlaagt wordt door volbloeddonatie via het onttrekken van cellen en plasmabestanddelen zoals eiwitten. Viscositeit is een bekende risicofactor voor de erosie en het scheuren van kwetsbare atherosclerotische plaques en de vorming van thrombo-embolieën. Omdat het aantal bloeddonaties geleidelijk aan de volbloed-viscositeit verlaagt, zullen de effecten op atherosclerose zoals in dit proefschrift beschreven grotendeels overeenkomen met die van een hypothetische studie naar viscositeit en atherosclerose. Wel zal het effect van viscositeit op de vorming van thrombo-embolieën nieuw onderzoek vergen.

Dit zou een alternatief causaal mechanisme kunnen zijn die de discrepantie in de resultaten van dit proefschrift verklaart.

Dit proefschrift duidt op een beschermend effect van volbloeddonatie tegen hart- en vaatziekten bij vrouwen, maar niet bij mannen. Het lijkt echter onwaarschijnlijk dat zo een beschermend effect het resultaat is van verbeteringen in het metabole spectrum en misschien zelfs ook niet van directe effecten op atherosclerose, maar eerder het gevolg is van een verlaagde viscositeit, of zelfs via een tot nog toe onbekend mechanisme. De volgende stappen zijn het onderzoek naar het onderliggende mechanisme hiervan, inclusief de geslachtsverschillen hierin, alsmede andere mogelijke langetermijneffecten van bloeddonatie.

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Curriculum Vitae

Karlijn Peffer was born on May 21st 1986 in Naarden, The Netherlands. She grew up in De Hilversumse Meent where she attended In de Kring (later known as de Sterrenwachter), after which she completed her secondary education at St. Vituscollege Bussum (atheneum). From 2004 until 2009, Karlijn studied Biomedical Sciences at the Radboud University Nijmegen. During her bachelor's in 2007, an internship on breast cancer mortality and adjuvant therapy introduced her to the field of epidemiology and Prof. dr. A.L.M. Verbeek. Recognising the importance of epidemiology as a fundament of clinical research, but attracted to the broad field and applicability of health technology assessment, she continued her master in both majors. In 2008, she conducted her Epidemiology internship at Sanquin Blood Supply on risk factors of turbid milky plasma donations, supervised by Dr. C.J.M. Doggen. For health technology assessment, an internship was completed in 2009 on Mindfulness-Based Cognitive Therapy under supervision of Prof. dr. A.E.M. Speckens. Upon graduation, she also completed the additional programme of the Nijmegen Centre for Evidence Based Practice. As of June 2015, she is appointed as an epidemiologist at Meander Medical Centre in Amersfoort.

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