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Markers of iron status and cardiometabolic disease risk: An exploration of the association based on cross-sectional and prospective studies in multiple populations

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Declaration of own work

I declare that this doctoral thesis is entirely my own work and has not been submitted for any other degree or qualification. Any publication derived from the chapters and mentioned as product of this research is my own work in terms of conception, data analysis and writing up of the first draft.

Signature _____ Date _____

Abstract

The aim of this thesis is to contribute to the understanding of iron metabolism, as a factor associated with cardiometabolic risk, by undertaking secondary data analyses. The objectives were to identify gaps in existing knowledge in terms of populations studied and alternative iron markers, and to attempt to fill the gaps with additional analyses and interpretation. Serum ferritin was the most widely available measure of iron status but the role of serum transferrin and soluble transferrin receptor (sTfR) levels was considered where available. I have taken a life-course approach with analyses in childhood and adulthood, and have included both intermediate factors such as the metabolic syndrome (MetS), and disease diagnoses of diabetes and cardiovascular disease as outcomes.

Chapter one presents a review of empirical research literature on the relationship between iron metabolism and cardiometabolic risk, concepts surrounding iron markers and the study outcomes. This chapter also describes the gaps in understanding the iron-cardiometabolic risk relationship, which are subsequently explored in chapters two to six.

Chapter two explores the link between serum ferritin and transferrin and MetS in cross-sectional and prospective studies of 725 Spanish children and 567 Chilean adolescents. I found associations between both ends of the ferritin distribution and MetS or glucose metabolism markers in different paediatric populations. For instance, whereas in the Spanish children there was a decrease of 0.02 SD units in the change of MetS score over time for every SD unit increase in ferritin, in the Chilean male adolescents being in the highest tertile of ferritin (v. the lowest) was associated with an increase of 0.25 SD units of MetS score. Furthermore, sustained high ferritin levels at various time points and gradual increase of ferritin during childhood were associated with higher MetS score in adolescence.

The third chapter describes the association between serum ferritin status and MetS in adults in two cross-sectional studies of Scottish populations (2,047 individuals from Shetland Islands and 8,563 subjects from the Scottish Health Surveys (SHeS) 1995-1998). I also examined the overall association between ferritin, MetS and each MetS

component in adults, by conducting a meta-analysis and investigating potential relevant sources of heterogeneity for the association. Interestingly, ferritin levels were positively associated with MetS in the Scottish populations, but the association was not independent of the effect of covariates, mainly body mass index (BMI) and transaminase levels [Men Odds ratio (OR) 95% confidence interval (CI) 1.43(0.83-2.46); Postmenopausal women OR (95%CI) 1.09(0.62-1.90); Premenopausal women OR (95%CI) 1.02(0.42-2.46), P>0.05]. The meta-analysis supported this finding by describing hepatic injury markers and BMI as the major attenuating factors of the ferritin-MetS association.

Chapter four investigates the association between sTfR or ferritin, and MetS in 725 Croatian adults in a cross-sectional study. There was no evidence of an association between sTfR and MetS [Men OR (95%CI) 1.35(0.90-2.02); Postmenopausal women OR (95%CI) 0.73(0.47-1.15); Premenopausal women OR (95%CI) 0.87(0.66-1.17), P>0.05]. In contrast serum ferritin, was positively and independently associated with MetS in men and postmenopausal women (P<0.05) [Men OR (95%CI) 1.78(1.31-2.42); Postmenopausal women OR (95%CI) 1.71(1.12-2.62); Premenopausal women OR (95%CI) 1.24(0.85-1.80)]. These contrasting results suggest that different iron markers reflect different physiological processes other than iron metabolism.

Chapter five evaluates the longitudinal association between serum ferritin and several cardiometabolic disease outcomes (CMDs) in the nationally representative SHeS 1995 and 1998 (n = 6,497). I found an independent positive longitudinal association between ferritin and cerebrovascular disease (CEVD), which was strengthened by using higher cut-points for increased ferritin [higher v. lowest sextile fully adjusted Hazard ratio(HR) 95%CI 2.08 (1.09-3.94), P=0.024], and a not significant association with coronary heart disease (CHD) after adjustment for covariates. My analyses confirmed the widely established association with type 2 diabetes (T2D) [whole sample fully adjusted HR 95% CI 1.59(1.10-2.34), P=0.006], even with serum ferritin within the normal range. The above set of observations confirm ferritin as biomarker mainly related to the development of T2D and identifies the need to investigate the association between ferritin and CEVD in other populations.

Chapter six investigates whether ferritin is associated with risk for cardiovascular complications among people with T2D using cross-sectional study designs in two populations with differing baseline cardiovascular risk (Spanish study SIDIAP n=38,617) and (Edinburgh Type 2 Diabetes Study (ET2DS) n= 821) with additional analysis of follow-up data for ET2DS. Interestingly, ferritin levels were negatively associated with prevalence of cardiovascular disease, mainly CHD, in people with T2D in both studies [ET2DS OR (95%CI): 0.80(0.67-0.96), P=0.020; SIDIAP study: 0.85(0.83-0.88), P<0.001). Ferritin was also negatively associated with incident cardiovascular disease in ET2DS: HR 95% CI: 0.39(0.16-0.93), P=0.035. Therefore, the association between iron status and CMD risk in people with T2D appears to differ from that in general populations in which a positive association has been more commonly described.

In conclusion, serum ferritin is associated with cardiometabolic risk in different ways in a variety of populations. Inconsistent associations for other iron markers suggest that iron biomarkers reflect factors other than iron homeostasis that influence cardiometabolic risk. The association between iron markers and MetS appears to differ between populations. This thesis illustrates the complex relationship between iron metabolism markers, MetS and CMD, and identifies the need for further research on the topic in order to extend knowledge about pathophysiology and the potential for measures of iron status as biomarkers for CMD.

Lay summary

The aim of this thesis was to help understand whether different measures of iron stores affect risk of metabolic syndrome (a combination of high waist measurement, high blood pressure, moderately high blood sugar levels below the level for diagnosing diabetes and abnormal blood fat levels), diabetes and cardiovascular disease. I used information collected in other research studies that had been done in children, adults and people who already had diabetes. Ferritin was the most widely available measure of iron status, but transferrin and soluble transferrin receptor (sTfR) levels were available in some studies. I found that there was a link between low ferritin and increasing cardiometabolic risk in children over time and that there was a link between high ferritin and higher cardiometabolic risk in adolescents. There was link between ferritin and metabolic syndrome in Croatian adults but no link was found in Scottish adults. I did not find a link between sTfR and risk of diabetes and cardiovascular disease in the Croatian study. Scottish adults with higher ferritin were more likely to develop diabetes during 14 years of follow-up than those with lower levels. People with diabetes with lower levels of ferritin were at higher risk of heart attack or stroke than people with higher levels. My findings show that these measures of iron status have a complicated relationship with risk of metabolic syndrome, diabetes and cardiovascular disease. Improving the understanding of this relationship may be helpful for understanding the cause of these common conditions.

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List of abbreviations

ApoB	Apolipoprotein B
ALT	Alanine-Aminotransferase
AMI	Acute myocardial infarction
ARIC	Atherosclerosis Risk in Communities Study
AST	Aspartate-Aminotransferase
ATP-III	Adult Treatment Panel III
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CEVD	Cerebrovascular Disease
CHD	Coronary heart disease
CMD	Cardiometabolic disease
DESIR	Data from an Epidemiological Study on the Insulin Resistance syndrome
DIOS	Dysmetabolic-hepatic Iron Overload Syndrome
EPIC	European Prospective Investigation into Cancer and Nutrition
GGT	Gamma-glutamyltransferase
HbA1C	Glycosylated haemoglobin
HBP	High blood pressure
HDL-C	High density lipoprotein Cholesterol

HFG	High fasting glucose
HH	Hereditary hemochromatosis
HOMA-IR	Homeostasis model assessment-Insulin resistance
hs-CRP	high sensitivity C reactive protein
HTG	High triglycerides
HWC	High waist circumference
IDF	International Diabetes Federation
IL-6	Interleukin 6
IQR	Interquartile range
IR	Insulin resistance
ISD	Information Services Division
IU	International units
KIHD	Kuopio ischemic heart disease study
KNHANES	Korea National Health and Nutrition Examination Survey
KORA	Cooperative Health Research in the Region Augsburg
LDL	Low density lipoprotein Cholesterol
MDA	Malondialdehyde
MONICA	Multinational Monitoring of trends and determinants in cardiovascular disease
NAFLD	Non-alcoholic fatty liver disease
NCEP	National Cholesterol Education Program

NHANES	National Health and Nutrition Examination Survey
MetS	Metabolic syndrome
PREDIMED	Prevención con Dieta Mediterránea
PURE	Prospective Urban Rural Epidemiology
RBP4	Retinol binding protein 4
SD	Standard deviation
SMR	Scottish Morbidity Record
SNP	Single-nucleotide polymorphism
T2D	Type 2 diabetes
TG	Triglycerides
TIBC	Total iron binding capacity
TfR	Transferrin receptor
TSAT	Transferrin saturation
sTfR	soluble transferrin receptor
VIKING	Viking Health Study – Shetland
WBC	White blood cells count
WC	Waist circumference
WHO	World health organization

Chapter 1

Introduction and overview of concepts, study variables, and rationale for general methodological approaches of the thesis

1.1 Introduction: General overview and framing of the PhD thesis

The aim of this thesis is to contribute to the understanding of iron metabolism, as a factor associated with risk of cardiometabolic disease (CMD), by undertaking secondary data analyses. In particular, the aim was to identify gaps in existing knowledge in terms of populations studied and alternative iron markers and to fill these gaps with additional analyses and interpretations.

This chapter presents the background to the thesis and includes biological and epidemiological observations which suggest that iron may be a causal factor in the development of type 2 diabetes (T2D) and cardiovascular disease (CVD). Evidence against the presence of a causal relationship is also considered. Although there were several longitudinal analyses in this thesis, the goal was to describe patterns of association rather than attributing causality. There still are multiple gaps in the knowledge on the topic, some of which will be addressed in further observational studies before considering whether the information add further support for the role of iron metabolism as a risk factor for CMD. I have taken a life-course approach with analyses in childhood and adulthood, including an intermediate risk factor, such as metabolic syndrome (MetS), and diabetes and CVD as outcomes, using longitudinal study designs where available. Serum ferritin was the most widely available measure of iron status but the roles of serum transferrin and soluble transferrin receptor (sTfR) levels were considered where data were available.

In addition, this introductory chapter provides the rationale behind the research questions and chapters of the thesis, with information about exposure and outcome variables, as well as the justification for methodological decisions.

1.1.1 Background to the research

Although the term "epidemic" is usually applied to infectious diseases, it has also been used to describe the increasing prevalence of CVD and diabetes, collectively described as CMD (1). Currently, ischemic heart disease and cerebrovascular disease (CEVD) are the top leading causes of natural death in the world, closely followed by diabetes and, according to projections for 2030, this scenario will not change (2). These diseases are attributed to the interaction between environmental and genetic factors that lead to arterial obstructions and alteration of glucose metabolism (3). Overweight/obesity, smoking, hypercaloric diets, and physical inactivity have been identified as well-known risk factors for CMD, and insulin resistance (IR) and resulting impairment of glucose metabolism have been implicated as a possible mechanism or earlier event (4). IR refers to the decreasing biological effect of insulin on tissues or organs sensitive to this hormone (liver, and adipose and skeletal muscle tissues) (5). Likewise, the cluster of metabolic, anthropometric, and vascular abnormalities, that is described as the metabolic syndrome has become an important tool to estimate the early risk of CMD and is associated with IR (6). However, CMD is a multi-factorial disorder and the exploration of potential new risk factors is necessary in order to obtain and a better understanding of CMD mechanisms and assessment of CMD risk.

The hypothesis that iron metabolism may be a potential risk factor for CMD in the general population arose from a variety of observations (7). In 1981, Sullivan suggested that lower iron stores may partly explain the lower risk of CVD in women than men. In addition, CVD and diabetes have been documented as frequent complications of hereditary hemochromatosis (HH), a genetic disease characterised by high body iron accumulation. Similarly, pathological conditions characterised by iron overload such as thalassemia are frequently associated with IR. Iron concentration is increased in thalassemia owing to therapy by multiple transfusions as supportive treatment, and patients with normal or impaired glucose tolerance have shown evidence of increased pancreatic β-cell activity, reflected by high levels of C-peptide, a structure present in the proinsulin molecule and released when insulin is synthesised (8).

In addition to inherent iron overload in the case of HH, dysmetabolic-hepatic iron overload syndrome (DIOS) is a kind of acquired iron overload (9). The main difference between HH and DIOS is that whereas in HH both transferrin saturation and serum ferritin are increased (> 45% and 300 μg/L respectively), in DIOS only ferritin levels are raised (10). This increased ferritin in DIOS is generally due to fatty liver and inflammation and patients with DIOS may present MetS features, hyperuricemia as well as altered levels of liver enzymes (9, 10).

A possible mechanism behind the relationship between iron excess and T2D and CVD is increased oxidative stress due to the pro-oxidant properties of iron (11, 12). The ferrous iron participates through the Fenton reaction in the formation of highly toxic free radicals as superoxide and hydroxyl anions, which can induce lipid peroxidation, damage in the mechanism of endothelial vasodilation, decrease in insulin signalling, and atherosclerotic plaque development (13, 14). In HH, the iron accumulation in the islets and pancreatic acini can directly damage insulin secretion because β cells are highly sensitive to free radicals (15). Moreover, an *in vitro* study reported the susceptibility of insulin to nitrosation by peroxynitrite (where free radicals are generated by combining nitric oxide with superoxide radicals); moreover, this structural alteration showed marked reduction in the hypoglycaemic effect of insulin in a group of monkeys who were administered the modified hormone, compared to the control group with unmodified insulin (16).

Of note, the relationship between iron and glucose metabolism is bidirectional (9). Insulin stimulates cellular uptake of many nutrients, including hexose sugars, amino acids, cations, and anions. This hormone causes a rapid and marked stimulation of the uptake of iron by adipocytes by redistributing TfR from the intracellular side of the membrane to the cell surface (11). These receptors are located along with insulin-dependent glucose transporters and insulin-like growth factor II receptors in the microsomal membranes of adipocytes, suggesting the regulation of iron uptake by insulin in addition to its known effects on glucose transport. Similarly, it has been found that insulin is responsible for the increased ferritin synthesis in cultured rat glioma cells (11). Recently, it was observed that gluconeogenesis triggered by

starvation induces iron deposition in the liver and stimulates hepcidin production – the hormone that down-regulates intestinal iron absorption (9).

Serum ferritin level is the most commonly used measure of iron stores and has been found to be associated with the risk of CMD in several studies (17-19). However, the association between measures of iron status and risk of CMD is still unclear. However no association between ferritin and risk of CMD in crude or adjusted analyses has been reported in other studies (20-22). Ferritin is an acute phase reactant, and inflammation is commonly associated with MetS and atherosclerosis and some studies suggest that inflammation rather than iron overload contributes to the association (23, 24). The role of chance, bias and/or confounding in the above discrepant findings will be considered later on in the respective discussion sections of chapters as well as in the conclusion chapter of this PhD thesis. Studies of the association between other measures of iron status such as transferrin and sTfR and risk of T2D and IR have also reported inconclusive findings. For instance, whereas a positive association has been described between serum transferrin and MetS in French and Swiss populations (7, 22), lower levels of transferrin in individuals with MetS have been described in people from an Italian village (25). Meanwhile sTfR levels were not found to be associated with incident T2D in English and Finnish cohorts (26, 27). In recent years, four meta-analyses have summarised and reported evidence of an association between ferritin and incidence of T2D (16-19). However the authors of the meta-analyses conclude that their findings may be affected by publication bias and unmeasured confounding, and that further research is needed to investigate whether there is a causal relationship between iron metabolism and incident diabetes.

Although iron excess is the most common derangement of iron status reported in relation to the risk of T2D and MetS, a systematic review of iron status and CVD showed that associations might exist with both iron excess and iron deficiency but they may be confounded by nutritional status (28). A similar U-shaped pattern for the relationship of iron status with mortality was observed among individuals with T2D and CVD. In 287 people with diabetes and stable coronary artery disease, both low and high serum ferritin levels were associated with 5-year all-cause mortality

(29). Studies involving animals and *in vitro* models are consistent with epidemiological observations. Iron deficiency has been associated with elevation in fasting blood glucose despite increased insulin sensitivity in animal models (30), and a dose-response relationship has also been described between anaemia and hyperglycaemia in rats (31). Similarly, iron deficiency has been found to be a promoter of increased lipid synthesis in the white adipose tissue of rats, enhanced expression of lipogenic genes, and dyslipidaemia (9). However, the specific mechanisms underlying the above findings are unclear (9).

The aim of this PhD is to describe the relationship between markers of iron status and risk of CMD (including MetS and its components, diabetes, and CMD) in order to address some of the gaps in existing knowledge.

1.1.2 Iron metabolism

Iron is a metal present in food in two forms: the divalent compound, ferrous, and the trivalent compound, ferric. The ferrous form is found mainly in meat and offal while ferric iron is primarily found in vegetables. Ferrous iron is highly soluble in gastric and duodenal juices and is the form in which iron can be absorbed by the intestinal cells, and only a small fraction of ferric iron can be converted to the ferrous form in the gut (32). Once iron is within the intestinal cells, it is again transformed into the ferric form and is bound by a circulating protein called transferrin, which transports iron to different tissues. In the tissues, iron plays a role in diverse metabolic functions and can be stored, mainly in the liver. In the bone marrow, it forms part of haemoglobin in the red blood cells. Old erythrocytes are removed from the circulation and broken down, releasing the iron, which is then recycled for new heme production, making iron metabolism very efficient (33).

Transferrin is a glycoprotein that transports iron in the blood and contributes to regulation of iron homeostasis. Transferrin has only two binding sites for ferric iron, and it can exist as apo-transferrin (no sites occupied), monoferric (single site occupied), or diferric form (both sites occupied with iron). The last form has the highest affinity for the TfR located in the cell membranes, whereas the monoferric and apo-proteic forms have intermediate and the lowest affinity for the receptor,

respectively (34). Diferric transferrin is recognised by a receptor in the liver, which stimulates the synthesis of the HFE (high Fe or human hemochromatosis protein), which in turn stimulates the hepatic synthesis and release of the hormone hepcidin (35). Hepcidin acts on intestinal cells to suppress the absorption of dietary iron in response to iron excess, mediated by elevated levels of diferric transferrin. In contrast, apo-transferrin and monoferric transferrin do not activate the above suppressor mechanism for absorption of dietary iron by intestinal cells (35).

Ferritin is an intracellular protein that stores iron and releases it in a controlled manner. Ferritin can bind approximately 4500 iron atoms and store iron in the ferric form (36). In addition, the storing of iron in the ferric form protects against the deleterious pro-oxidant effects of ferrous iron. Ferritin production increases with increasing absorption of dietary iron (36).

Ferritin is also an acute phase reactant with levels increasing in the presence of inflammation/infection (23). In contrast, transferrin levels decrease during acute phase reactions (37). This is related to a survival mechanism in situations of infection, since iron sequestration by ferritin and the suppression of iron transport in the bloodstream prevents pathogens from using iron for their metabolism and growth (36).

In summary, the different iron markers play the following roles in iron metabolism:

Ferritin: iron storage

Transferrin: transport of iron in the bloodstream

Transferrin receptor: transport of iron bound to transferrin into the cells. Hepatic TfRs are involved in sensing iron status – the first step in modulation of intestinal iron absorption

Hepcidin: suppression of intestinal iron absorption in response to a low iron demand (adequate or excess iron stores)

Although ferritin and TfRs do not circulate in the bloodstream, soluble fractions of these proteins are released into the circulation, which can be measured and used to represent measures of ferritin and TfRs (with the latter known as sTfRs)(38, 39).

Iron markers show opposite patterns in conditions of iron deficiency and iron overload as follows:

> Iron deficiency results in low ferritin and high transferrin and sTfR levels. When cellular iron levels are low, storage of iron by ferritin is not possible because the available iron is quickly metabolised. Therefore, the levels of transferrin and sTfR are increased in compensation to transport and take up as much iron as possible into the cells, which are in a high demand state (35).
>
> Iron excess or overload results in high ferritin and low transferrin and sTfR levels. In the case of iron excess, the ferritin level increases to store the residual iron not needed for metabolic functions. Meanwhile, the levels of transferrin and sTfR decrease because the cellular demands are low (35). There is an increase in hepcidin in response to cellular iron overload and a decrease in high demand status, such as iron deficiency. However, the levels of hepcidin decrease when iron excess is the result of classic HH, in which mutations occurs in the *HFE* gene preventing hepcidin increase.

1.1.3 Concepts and definitions of outcome variables

In this PhD thesis, the main outcome variables are MetS, diabetes, and CVD. MetS components, IR and glycosylated haemoglobin, are variables that are closely linked to risk of CMD; findings on these additional outcomes will be presented in the appendix as supplementary material. The definitions and epidemiological data related to the outcomes are given below.

Metabolic syndrome

According to the joint statement by several associations (40), MetS is defined as a "complex of interrelated risk factors for CVD and diabetes. These factors include dysglycemia, raised blood pressure, elevated triglyceride levels, low high-density

lipoprotein cholesterol levels, and obesity (particularly central adiposity)". MetS is also frequently associated with prothrombotic and proinflammatory states (41).

Appendix Table 1 presents the definitions and criteria of MetS for adults, as specified by several organisations. The different definitions require the presence of three out of the five factors or components to meet the criteria for MetS. Differences in MetS definitions arise from the differences in cut-off points for MetS components or components mandatorily required to define MetS, such as central adiposity in terms of increased waist circumference (WC) in the case of the International Diabetes Federation (IDF) definition.

In particular, the WC criterion has been the most difficult to reach consensus on owing to ethnic variations in adiposity distribution in addition to sex differences. The joint statement definition, also known as the harmonised definition, addressed this issue by defining different cut-points for increased WC according to ethnicity and geographical location as well as for men and women (40).

The concept and clinical utility of MetS has been controversial within the diabetes and endocrine research community. For some academics, although MetS is recognised as a cluster of cardiometabolic alterations, it lacks a core feature linking these alterations (42). IR has been proposed to be that feature, but this view has its criticisms as IR is difficult to estimate and differentially related to the individual MetS components (42). Despite the debate, MetS has been found to increase the risk of CVD by two-fold according to a meta-analysis of 87 studies (951,083 subjects) (41). However, there is still a need for large studies and meta-analyses on the risk of CMD conferred by the cluster and each of its individual components to determine whether there is a greater risk posed by the cluster. Those who defend the clinical utility of MetS, view the cluster as a factor for calling attention to patients at increased risk of CMD that might benefit from prophylactic treatments and as a stimulus for additional research on the etiological mechanisms of the cardiometabolic alterations and therapies to simultaneously reduce multiple risk factors (43). Given the above controversy, this PhD thesis investigated the association of measures of iron status with the cluster and also with each individual MetS component.

The prevalence of MetS in the world is highly variable and ranges between less than 10% up to 84%, depending on different factors such as the definition used, sex, age, race, and ethnic distribution of the population studied (44). The IDF has estimated that around 25% of the world population has MetS (44). MetS is more prevalent in overweight people than in those with normal weight (45). A review comparing studies which used the ATP-III definition for MetS, highlighted that the prevalence of MetS is higher in US (23.7–34.6%) than in European populations (10–23.6%) (45). the same review reported that two Latin-American studies reported high MetS prevalence (31.2% and 35.1%) (45).

Diabetes mellitus

A report by the World Health Organization (WHO) defines diabetes mellitus as "*a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both*. *Type 1 diabetes encompasses cases which are primarily due to pancreatic islet beta-cell destruction and are prone to ketoacidosis. Type 2 includes the common major form of diabetes, which results from defect(s) in insulin secretion, almost always with a major contribution from insulin resistance*" (46).

The global prevalence of diabetes was estimated to be 2.8% in 2000 and 6.4% in 2010 with prevalence projections for 2030 between 4.4% and 7.7% (47, 48). In Scotland, there was an increase in T2D prevalence between 2004 and 2015 (3.2% to 5.1%), which was largely explained by the reduction in mortality over this period rather than by increases in incidence (49). According to the Diabetes Scotland report of 2015, it has been estimated that around 500,000 people are at high risk of developing T2D, and 480,000 people in Scotland will be living with diabetes by 2035 (50).

Cardiovascular diseases

In this thesis, only ischaemic heart disease, i.e. history of angina pectoris or heart attack, and CEVD, i.e. transient ischaemic attack or stroke (ischemic and/or haemorrhagic), were considered as CVD outcomes because these are the major forms of the disease. According to the WHO, coronary or ischaemic (or ischemic) heart

disease "*is characterized by reduced blood supply to the heart caused by disease of the blood vessels supplying the heart muscle (51). The two leading manifestations of ischaemic heart disease are angina pectoris and acute myocardial infarction (AMI). Angina pectoris is defined by the WHO as a pressure-like pain in the chest that is induced by exertion or stress and relieved within minutes after cessation of effort or using sublingual nitroglycerin. Unstable angina represents a spectrum of clinical states that fall between stable angina and acute myocardial infarction (MI). Chest pain is subjective and there is no 'gold standard' for estimating the prevalence of angina in populations. Unstable angina is generally associated with hospital admission in developed countries but stable angina is managed in the community*" (51).

For stroke, the WHO has the following definition: "*rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin*" (52). Transient ischemic attack (TIA) is defined in a similar way but with a duration of less than 24 hours (52).

According to the WHO, in 2012, CVD and coronary heart disease (CHD) were estimated to account for 31.4% and 13.2% of global deaths, respectively (53). For 2030, CEVD is expected to account for 10.6% of deaths in the world (2). Age-sex standardised mortality rates per 100,000 inhabitants for all heart diseases in Scotland were around 300 in 2006 and 200 in 2014 (54). Despite this reduction in mortality over time, the prevalence of CHD in Scotland has been stable between 2004/2005 and 2014/2015 at approximately 4%, and the prevalence of stroke has been around 2% during 2003–2013 but with a slight increase from 2010 till 2013 (55). Within the UK, in 2014, Scotland had the highest prevalence of CVD (4.3%), second to the North of England (4.5%) (56).

Insulin resistance

As mentioned previously, IR is defined as the reduced biological effect of insulin on tissues or organs that normally respond to this hormone (5). The main biological effect of insulin is to stimulate glucose uptake by tissues and thus decrease the circulating levels of carbohydrates. IR is initially characterised by compensatory

hyperinsulinemia, given the decreased glucose uptake in the tissues (5). The "euglycemic-hyperinsulinemic clamp" is currently considered the "gold standard" for estimating IR and consists of intravenous administration of high doses of insulin and glucose infusion to maintain steady blood glucose levels within the normal range, according to the periodic monitoring of blood glucose in response to insulin (57). The glucose infusion rate is the evaluation parameter and the higher it is the greater the insulin sensitivity (57). However, this technique is invasive and expensive, requires training and a specialist software, and the patient requires hospital stay of around three hours for the procedure (57). Another method is the "short insulin tolerance test" in which a baseline blood sample and five samples after an insulin injection are taken at intervals of three minutes. The evaluation parameter in this short test is the slope of a graph of glucose concentration against time, with slopes closer to zero indicating increased IR (58). In 1985, Mathews et al., proposed an equation using an iterative testing model called Homeostatic Model Assessment Insulin Resistance (HOMA-IR) in which higher values reflect greater IR. HOMA-IR is calculated by multiplying the basal concentrations of glucose and insulin (mIU/L), and dividing this product by 22.5, if the glucose concentration is in mmol/L or by 445 if it is in mg/dL (59). HOMA-IR has been validated against estimates of insulin sensitivity derived from clamps in several studies, and estimates of IR have shown good correlation (Spearman coefficient= -0.85, $P < 0.0001$ (60); $r = -0.725$, $P < 0.0001$ (61)). Because of its practicality, the HOMA-IR index has been used in many research studies as an estimate of IR, including data sets described in this thesis in which relevant information was available.

Glycosylated haemoglobin

Glycosylated haemoglobin, also known as glycated haemoglobin, glycohaemoglobin, HbA1c, HbA1, or A1C, consists of haemoglobin portions formed from haemoglobin and glucose in a non-enzymatic way during a slow process (62). While blood measurements of glucose provide immediate information on glucose levels, glycosylated proteins, such as HbA1C, give long-term information on glycaemic history of around 120 days (62). HbA1C is commonly used as a marker for diabetic control, and compared to fasting glucose, it has shown similar predictions for risk of

diabetes and stronger association with risk of CVD in the general population (63). Increasing erythrocyte lifespan can increase HbA1C values. The lifespan of erythrocytes increases in situations of decreased erythropoiesis as a consequence of iron or vitamin B12 deficiency, lack of erythropoietin in renal failure, and bone marrow suppression in pregnancy and alcoholism (64). In contrast, situations of haemolysis lead to decreased values of HbA1C (64). An HbA1c of 6.5% is recommended as a cut-off point for diagnosing diabetes (65). A value of less than 6.5% does not exclude diabetes diagnosed using glucose tests (65).

1.2 Overview of and rationale for the thesis chapters

As mentioned previously, this thesis evaluates the association between levels of iron metabolism markers and cardiometabolic risk using both cross-sectional and longitudinal study designs in a variety of populations. The life-course approach is reflected in the use of data for both children and adults to investigate whether the association patterns are consistent. The outcomes include MetS and its components as risk factors for CMD as well as CMDs. Figure 1 depicts a general scheme of the thesis, which shows gaps in knowledge on the association of ferritin levels (and other iron markers) with MetS, its components, T2D and CVD, that I investigated in this thesis.

Chapter 2 describes the association patterns between iron markers and MetS in paediatric populations. It is well known that the risk for developing CMD starts in early stages of life, and prevalence and consequences of risk factors such as hypercaloric diet, obesity, and physical inactivity have been widely explored in children. In the light of the relevance of early detection of CMD, it is also important to investigate the effects of potential new risk factors or biomarkers identified in adults, such as iron metabolism, among children. So far, there is little information available on the association between iron metabolism and metabolic profiles in children. This chapter consists of cross-sectional and longitudinal analyses in two cohorts of Spanish and Chilean children. The analysis involving Spanish children provided the opportunity to study both serum transferrin and ferritin levels as

exposures, and the Chilean cohort provided the opportunity to evaluate repeated measurements of ferritin throughout 11 years' follow-up as the exposure.

The third chapter of this thesis includes cross-sectional studies investigating the association between serum ferritin and MetS and its components in two Scottish adult populations and a systematic review/meta-analysis of that association including the findings of the Scottish studies. These are the first studies to investigate the ferritin-MetS association in British or Scottish populations. There are two meta-analyses on this association, but neither of them focused on the associations with individual MetS components. Between 2014 and 2015, several new articles on the topic not included in the previous meta-analyses were published. In addition, there is a dearth of narrative syntheses of the studies, and the potential sources of heterogeneity have not been studied well enough. Hence, the systematic review/meta-analysis is an attempt to cover these aspects.

The cross-sectional association between serum TfR levels (sTfR), MetS and its components, and IR in an adult population is presented in Chapter 4. There are few other studies of sTfR as the iron marker that have investigated the association with cardiometabolic risk factors. The chapter uses data from a Croatian population and also describes the association between serum ferritin and MetS.

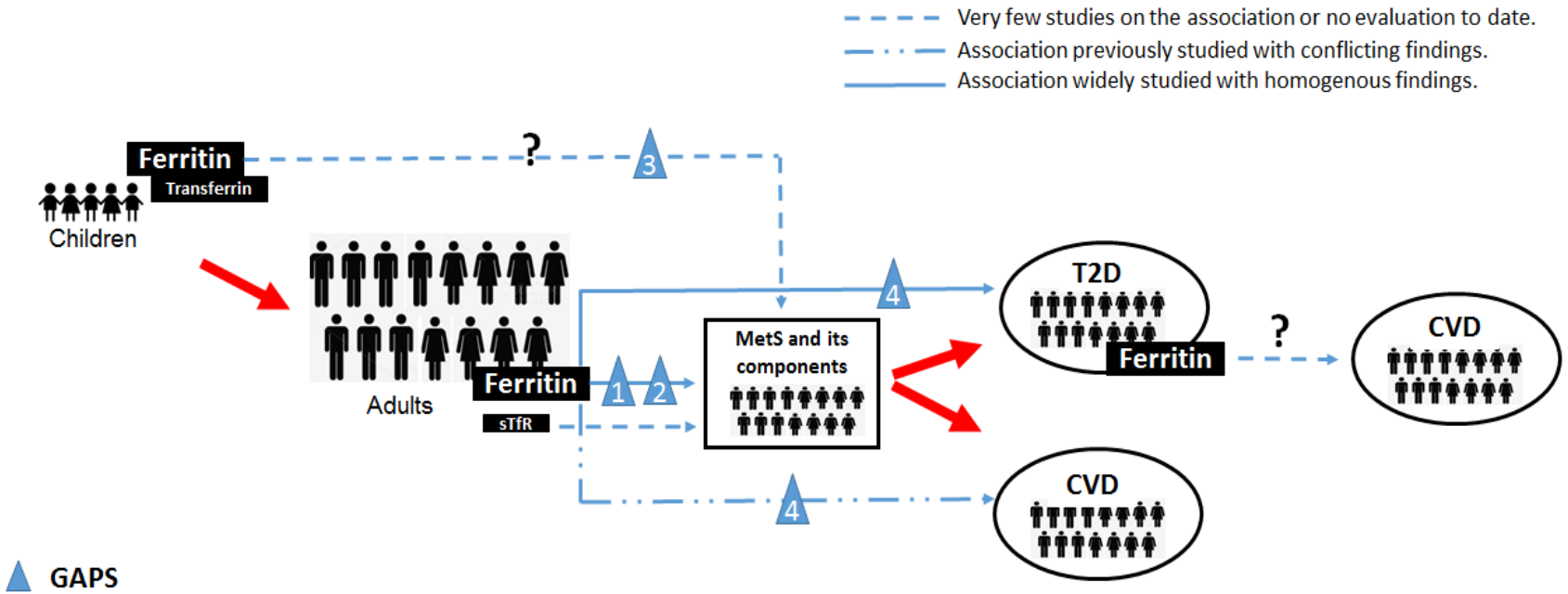
Chapter 5 comprises longitudinal analyses, describing the association of ferritin with the incidence of diabetes and CVD, using data from Scottish Health surveys 1995-1998 linked to the national diabetes register, hospital admission and mortality records. No previous similar studies have been performed in Scottish populations and no previous studies have simultaneously described the associations between ferritin and outcomes of T2D, CHD, and CEVD; there is very little information on the association between ferritin and CEVD in particular.

Chapter 6 describes the studies on the association between iron status and cardiovascular complications in people with T2D using data from the Edinburgh Type 2 Diabetes Study and the SIDIAP study (data from primary health care centres

in Catalonia). Little attention has been given to associations between iron status and complications of diabetes. If iron metabolism is a causal factor or one of several metabolic abnormalities implicated in the development of CMD in general populations, iron misbalance could potentiate the risk of cardiovascular outcomes in people who already have diabetes. I have not identified any previously published similar large or longitudinal studies that have used multivariable adjustment.

Chapter 7, the concluding chapter, collates the information presented in the earlier chapters and discusses potential interpretations of the findings in light of the strengths and limitations of the work. Recommendations for future studies and potential priorities for research are also discussed here.

Figure 1 General scheme of research questions in the thesis



1.3 Rationale behind general methodological approaches in this thesis

1.3.1 Choice of iron markers

In this PhD thesis, I have focused on three serum proteins – ferritin, sTfR, and transferrin – with different functions in iron metabolism - as the exposures of interest. There are other iron markers such as total iron binding capacity (TIBC) and transferrin saturation percentage (%TS). I identified the following disadvantages of these additional iron markers which is why I excluded them from further study:

- Estimation of TIBC and transferrin saturation require the measurement of serum iron, a marker susceptible to diverse sources of error such as incomplete dissociation of iron from binding proteins; loss during the precipitation process; and interferences by copper, haemoglobin, bilirubin, and lipids (66). TIBC and serum iron have also shown greater between-day variation than other iron markers (67).
- TIBC and %TS are less commonly measured and it is common to find more missing values for these iron markers even in databases where the variables are included, as laboratory techniques to measure them require larger amounts of blood serum samples than for serum iron proteins.
- Although having as many iron makers as possible could be useful in terms of comparing and contrasting specific findings for different markers, this also further increases the potential for type 1 errors as a consequence of multiple testing.

1.3.2 Definition of exposure variables

Serum levels of ferritin, transferrin, and sTfR were used as continuous variables and as categorical variables by using quantiles to explore linear and non-linear relationships. Tertiles were used when samples by sex and/or menopausal status were smaller than 500 individuals to facilitate better comparability between the highest and lowest categories. Quartiles of the iron markers were used when sub-groups were larger than 500. In Chapter 6, quintiles, not quartiles, of ferritin were shown because

by using quintiles, the longitudinal association evaluated was clearer. The implications of this pattern are discussed.

1.3.3 Choice of covariates

In most of the analyses reported in this thesis, the adjustment variables included age, sex, menopausal status, body mass index (BMI), markers of clinical/subclinical inflammation and hepatic function, smoking status, alcohol consumption, and presence of CMD, where appropriate.

Markers of iron status, such as ferritin, vary across different life stages. Iron stores are high in new-borns until the first two months and decrease during infancy. Ferritin levels rise again at adolescence, and peak concentrations for men have been identified between 30 and 39 years (68). Aging has been linked to decrease in ferritin presumably due to a combination of physiological and pathological factors (69).

Iron status differs markedly by sex and menopausal status with lower levels of iron stores found in women than men and in premenopausal than post-menopausal women (70). This implies the potential for confounding or effect modification. Therefore, the descriptive and analysis tables from each study included in the thesis present either results stratified by groups of sex/menopausal status or by sex-menopausal specific quantiles of the iron markers.

The remaining set of confounding factors was chosen on the basis of the possible association of acute phase or subclinical inflammation (discussed above), adiposity, and liver dysfunction with both the levels of iron markers and outcome variables.

Several studies have reported associations between markers of subclinical inflammation and liver function such as transaminases with cardiovascular risk factors, risk of diabetes, and CVD (71, 72). Since ferritin, sTfR, and transferrin are synthesised in the liver, hepatic dysfunction can lead to an increase in their circulating levels owing to destruction of hepatic cells (73). Besides its well-known association with higher cardiovascular risk, adiposity can influence the markers of

iron status. The higher the adipose mass the higher the iron stores, since adipocytes contain ferritin (74). Adiposity can also increase ferritin levels via subclinical inflammation induced by inflammatory cytokines released by adipose tissue (75). However, obesity-related chronic inflammation can less commonly lead to iron deficiency because high levels of circulating ferritin have an inhibitory effect on intestinal iron absorption (75).

Smoking and alcohol consumption are common adjustments in analyses with cardiometabolic outcomes (76, 77). These lifestyle variables are important potential confounders owing to their association with both CMD risk factors and MetS components. In contrast, there is no major evidence of the association between smoking and alcohol intake and iron markers. However, alcoholic liver disease may lead to increased levels of ferritin.

Although physical activity and caloric intake influence the risk of CMD, they were not included as main covariates owing to the potential for over-adjustment, given their effect on BMI (78). BMI appears to be more strongly associated with CMD than physical activity (79), and overweight/obesity may promote physical inactivity (80). Physical activity and caloric intake are also extremely difficult to measure reliably (81, 82). In addition, the nutritional epidemiology course I attended in 2015 recommended minimising the number of covariates and avoiding or minimising adjustments for inter-related covariates.

Where available, adjustment for T2D and CVD was conducted for the iron markers-MetS association, as a sensitivity analysis. This is because established CMD could influence iron marker levels and create the potential for reverse causality.

1.3.4 Metabolic syndrome and assessment of prevalence in adults and children

There are several definitions of MetS in adults based on clusters of MetS components based on levels of selected cardiovascular risk factors above or below the specific cut-off points. For the analyses I conducted this PhD thesis, I adopted the harmonised definition for MetS in adults which was agreed upon in 2009 by a group of

international endocrinology and cardiovascular research associations (40). In contrast, there is no consensus on the definition of MetS for children and adolescents. There are some proposed definitions for children older than 10 or 12 years using categorical variables of risk factors, similar to those used for adults (83-85). The use of these definitions in cross-sectional studies involving children results in lower statistical power since MetS and most of its components defined using categorical variables tend to have a low prevalence in paediatric populations. As the studies in my thesis included children younger than 10 years and since I needed to ensure sufficient statistical power, I decided to use continuous variables on the basis of Z scores to describe MetS and its components. This approach has been used previously for research on cardiovascular risk in childhood (86) and it is described further in the relevant chapter.

1.3.5 Multiple testing

Every analysis in this PhD thesis describes associations of iron markers with at least three or more outcomes. The analyses with more outcomes are those in which associations with the five MetS components are also evaluated. This multiple testing might lead to "false positive" associations, mistakenly rejecting the null hypothesis as a result of type 1 errors. However, methods for correction of P values for multiple testing using Bonferroni adjustments were not used in the present thesis. The following bullet points based on some previous criticisms for such corrections provide support to this approach:

-Bonferroni adjustment is focused on the null hypothesis, which is rarely of interest to the researchers (87). These adjustments are intended to minimise type I errors but at the expense of increasing the likelihood of type II errors or false non-significant associations, rejecting the alternative hypothesis (87, 88). For instance, MetS and its components form a special case as a group of outcomes since MetS is a cluster of metabolic alterations, but it is possible that each metabolic alteration or component could have an association pattern with the exposure variable that differs from that of the cluster. I decided that dividing the common accepted level of significance, 0.05, by the number of tests conducted, as Bonferroni adjustments demand, was too

conservative for exploratory analyses such as those conducted in this PhD thesis. For instance, if taking into account a total number of 18 tests as a result of six associations evaluated (MetS and its five components) in three sub-groups of sex and menopausal status, a P value lower than 0.002 would be set as significant. The requirement for significance would be even lower and difficult to satisfy if explorations with different iron markers, kind of exposure variable (continuous and categorical), are also considered in the number of tests.

-The interpretation of Bonferroni-corrected p values is based on how many tests are conducted, which is irrelevant in clinical practice. For example, abnormal or normal levels of a biomarker would not be determined depending on whether levels of other biomarkers were also tested in the same individual (87). Another very important weakness of the Bonferroni approach is that it is difficult to have a reliable estimate of the number of tests performed. The association between a diverse and large number of exposure variables and a single outcome may be performed by different researchers throughout the existence of the dataset (87).

In light of the above points, this PhD thesis reports non-corrected p values for the associations reported to avoid missing important findings and the maximum probability to accept a false positive finding was set as 0.05, by using two-tailed significance tests, given that either iron deficiency or iron excess could be associated with the outcomes. The associations obtained are discussed according to the coherence and plausibility of the results, considering the potential impact of multiple testing and recommending replication in future studies.

This chapter has covered aspects of iron metabolism as well as concepts on outcome variables of this thesis work. Similarly, it has provided the rationale for the general methodological decisions throughout the different analyses in the chapters. The next chapter will describe the association between iron markers and MetS in two cohorts of children and adolescents.

Chapter 2

Iron markers and cardiometabolic risk in paediatric populations

2.1 Introduction

To date, little is understood about whether there is an association between iron and cardiometabolic risk in early life. Children have a lower capacity for iron storage than adults since this physiological function becomes fully developed in adulthood. It is not known whether changes in iron stores across infancy and adolescence are associated with MetS and if these changes could have an additive effect on cardiometabolic risk. Whether iron excess, iron deficiency, or both are involved in higher cardiometabolic risk during childhood is also unknown. Therefore, two studies were conducted in paediatric cohorts from Spain and Chile to evaluate cross-sectional and longitudinal associations between ferritin levels, MetS, and its components. In addition, associations with serum transferrin were also studied in the Spanish cohort, and the relationship between changes in ferritin during childhood and MetS risk in adolescence were investigated in the Chilean cohort.

2.2 Methods

2.2.1 Subjects

The present chapter used data from two cohorts: Spanish and Chilean children. The Spanish cohort consisted of school-aged children of European ancestry aged 3.4–14 years old (8.2 ± 2.0 years) who were included in a cross-sectional study of cardiovascular risk factors in prepubertal children. The subjects were consecutively recruited from among those seen in the primary care setting in the Alt Empordà and Girona regions in north-eastern Spain between 2009 and 2014. Participation ranged from 50% to 70% among the different centres. Of the 832 participants whose data were analysed at baseline, 207 children [94 boys and 109 girls] were studied again after a mean follow-up of 3.7 ± 0.8 years [range 2–6]. For the analyses of this chapter, the final sample after removing cases with missing values for

exposure/outcome variables or covariates consisted of 726 subjects at baseline (376 boys and 350 girls) and 173 (83 boys and 90 girls) with follow-up data. Inclusion criteria of the original study at baseline were 1) European ancestry; 2) age between 5 and 14 years; and 3) no pubertal development, as judged by a specifically trained nurse using Tanner criteria (breast stage I; testicular volume < 4 mL) (89, 90). To avoid findings biased by acute or chronic illness, the exclusion criteria were as follows: 1) major congenital anomalies; 2) abnormal blood count and liver/kidney/thyroid functions; 3) evidence of chronic illness or prolonged use of medication; and 4) acute illness or use of medications in the month preceding potential enrolment. The study protocol was approved by the Institutional Review Board of Dr. JosepTrueta Hospital. Signed consent for participation in the study was obtained from parents.

The Chilean cohort included infants of low/middle socio-economic status from urban Santiago recruited between 1991 and 1996 for a trial of iron supplementation. The infants, recruited at 4 months, were healthy, full-term singleton infants weighing 3 kg or more at birth. At 6 months, those who did not have iron-deficiency anaemia were randomised to receive iron supplementation or usual nutrition between 6 and 12 months. The cohort was assessed for developmental outcomes, including ferritin levels, in infancy and at 5, 10, and 16 years (91). At 16 years, participants were also assessed for obesity and cardiovascular risk (92). Three samples from the cohort were evaluated: 1) participants with no missing values for ferritin and covariates at 5 years and for cardiometabolic risk outcomes at 16 years (n = 565); 2) participants with no missing values for ferritin and covariates at 10 years and for cardiometabolic risk outcomes at 16 years (n = 381); and 3) participants with no missing values for ferritin, covariates, and cardiometabolic risk outcomes at 16 years (n = 567). A sample with no missing values for the study variables at any stage of the follow-up was also evaluated (n = 379). The study was approved by the institutional review boards of the University of Michigan, the Institute of Nutrition and Food Technology (University of Chile), and the University of California, San Diego. Participants and their primary caregiver provided informed and written consent, which was obtained

according to the norms for Human Experimentation, Code of Ethics of the World Medical Association (Declaration of Helsinki, 1995).

2.2.2 Clinical assessments

Standardised procedures were conducted to measure the height to the nearest 0.1 cm using Harpenden (Spanish Cohort) and Holtain (Chilean Cohort) stadiometers and weight to the nearest 0·1 kg using a Seca scales in the Spanish and Chilean cohorts. The BMI (kg/m^2) was then calculated as weight divided by the square of height. WC was measured with a non-elastic flexible tape at the high point of the iliac crest around the abdomen and recorded to the nearest 0.1 cm. Measurements were taken twice, where a third of the measurements of the difference between the first two exceeded 0.3 kg for weight, 0.5 cm for height, and 1.0 cm for the waist. Age- and sex-adjusted Z-score values for BMI were calculated using regional normative data for the Spanish children and the CDC reference for the Chilean cohort (93, 94). Categories of weight status were defined according to BMI Z score cut-off points as follows: underweight, ≤1; normal weight, -1 to 1; overweight, >1 and <2; and obese, >2 (95).

Blood pressure (BP) was measured in the supine position on the right arm after a rest period of 10–15 min; an electronic sphygmomanometer (Dinamap Pro 100; GE Healthcare, Chalfont St. Giles, United Kingdom) with cuff size appropriate for arm circumference was used in the Spanish cohort, while for the Chilean cohort, standard mercury sphygmomanometers were used. In both cohorts, averages of three readings taken at 5-min intervals were recorded for each subject.

Fasting serum total glucose, cholesterol, triglycerides, HDL-cholesterol (HDL-C), insulin, adiponectin, and high-sensitivity C-reactive protein (hs-CRP) levels were measured after a 12-h overnight fast. Insulin levels were measured by immunochemiluminescence in the Spanish cohort (IMMULITE 2000; Diagnostic Products, Los Angeles, CA) and by RIA in the Chilean cohort (Diagnostic Products Corporation, Los Angeles, CA, USA). Also, hs-CRP level was measured with a sensitive latex-based immunoassay in both cohorts. In the Spanish cohort, serum

glucose level was measured using the hexokinase method, HDL cholesterol, using the homogenous method of selective detergent with accelerator, and serum triglycerides, using the glycerol-phosphate-oxidase and peroxidase methods. In the Chilean cohort, glucose level was measured with an enzymatic colorimetric test (QCA S.A., Amposta, Spain) and cholesterol profile (HDL-C and triglycerides, mg/dL) was determined by dry analytical methodology (Vitros®; Ortho Clinical Diagnostics Inc., Raritan, NJ, USA). Glycosylated haemoglobin (HbA1C), available in the Spanish cohort, was measured by high-performance liquid chromatography (Bio-Rad, Munich, Germany) and a Jokoh HS-10 autoanalyser. In the Spanish cohort, serum alanine transaminase (ALT) and gamma glutamyltranspeptidase (GGT) were measured by colorimetry using automated tests, and serum transferrin by routine laboratory tests (Beckman, Fullerton, CA). Serum ferritin was measured by microparticle enzyme immunoassay (AxSYMTM; Abbot Laboratories, USA).

In the Spanish cohort, anaemia was defined as haemoglobin <11g/dL for children <5 years, < 11.5 g/dL for children 5 to 11 years, and < 12 g/dL for children aged 12 years and older (96). Serum ferritin < 7 μg/L was categorised as iron deficiency (97).

2.2.3 Metabolic syndrome and insulin resistance

As mentioned in chapter 1 I decided to evaluate continuous MetS scores instead of using paediatric MetS definitions. The latter approach would have had lower statistical power as few cases met the categorical criteria from MetS definitions (e.g. high glucose as glucose > 100 mg/dL). Similarly, there is no consensus on the cut-off values for the different components of MetS in children and adolescents. A continuous variable of Z score or scale of SD units for MetS was created from the average of Z scores of blood pressure, glucose, triglycerides, HDL cholesterol, and WC (86). The distribution of these MetS components was normalised, if required, before calculating the Z scores. Details on these transformations are provided in the data analysis section of each cohort. The Z scores of diastolic and systolic blood pressures (DBP and SBP, respectively) were averaged to get a single Z score for blood pressure, and the HDL-C Z score was multiplied by -1 before obtaining the overall MetS score to ensure that all of the components of MetS had the same

positive association with regard to cardiometabolic risk. IR was estimated by using the formula of the HOMA-IR as: (glucose[mg/dL] × insulin [mU/mL])/44.5(59).

2.2.4 Data analysis

The analyses were conducted with female and male subjects separately, given the differences by sex in cardiometabolic outcomes in both cohorts. The study variables were described as median (and interquartile range) and proportion, and the differences were estimated by Mann-Whitney *U* test and Chi-square test. Linear regression analyses were used to evaluate the association between the iron markers and the MetS Z score in both cohorts. In the Spanish cohort, continuous exposure and outcome variables were used to compensate for the limited statistical power in the prospective analysis. In the Chilean cohort, the exposure variable of ferritin was used as tertiles and the outcome, MetS score, as continuous. This categorisation of ferritin aimed to enable a comparison of associations at single time points with associations on patterns of sustained high/high vs. low/low ferritin during the follow-up of this cohort (more details below).

Association analyses: Spanish cohort

Multivariate linear regression analyses were conducted by sex and in the whole sample to evaluate and adjust cross-sectional associations of age (quintiles)/sex-specific Z scores for ferritin and transferrin (exposure variables) with the age/sex-specific Z scores of MetS and MetS-related variables (SBP, DBP, WC, glucose, triglycerides, and HDL-C), HbA1C, and HOMA-IR at baseline (outcome variables). Subclinical inflammation and/or infection, hepatic disorders, and overweight/obesity are associated with increased ferritin levels and are associated with an increase in cardiometabolic risk markers and could confound any association. Therefore, the regression coefficients were adjusted for the above-mentioned potential confounders in terms of age, CRP levels, ALT and GGT levels, BMI Z score, and also sex (for analyses of the whole sample). Relationships with SBP and DBP were additionally adjusted for height since this variable has an influence on blood pressure during growth. Since outcome and exposure variables were standardised by quintiles of age, I also compared models with and without age (continuous variable) as covariate to

identify any effect of this adjustment. Changes per year of follow up in MetS, MetS-related markers, and HOMA-IR were calculated as: (measurement 2 - measurement 1)/years of follow-up. Associations between Z scores of the iron markers at baseline and the change per year in the score for MetS-related markers and HOMA-IR, adjusting for covariates at baseline, were estimated using linear regression. The adjustment in these longitudinal associations also included baseline values of the outcomes (e.g. the associations between ferritin Z score and change in MetS score was further adjusted for MetS Z score at baseline). This approach was chosen as the baseline values may have some influence on the change outcomes according to a weak correlation between the change in MetS Z score and the average calculated from MetS score at baseline and MetS score after follow-up (Spearman coefficient 0.176, P = 0.021)(98). Before calculating the Z scores (ferritin and MetS components) and conducting the analyses, skewed variables were log-transformed [ferritin, WC (baseline), triglycerides CRP, ALT, GGT] to approximate to the normal distribution, except CRP levels which were normalised using the natural logarithm of 1/CRP.

Association analyses: Chilean cohort

Ferritin levels were defined in tertiles at 5, 10, and 16 years for participants with data available at that time point. Patterns of ferritin levels were created from different combinations of high ferritin (highest tertile) and low-moderate ferritin (lowest and middle tertile) for intervals 5 and 10 years, 10 and 16 years, and 5 and16 years. Possible patterns were the following: high/ high (or persistently high), high/ low-moderate, low-moderate/ high, and low-moderate/low-moderate (or persistently low-moderate) which was the reference category for comparisons. Patterns of ferritin using the three time points were additionally calculated but assuming low number of cases for some categories (high/high/high, high/high/low-moderate, and so forth). I also calculated the slope of ferritin concentration per year (99) and used this parameter as continuous and categorical variable (tertile). Multiple linear regression was used to evaluate associations of ferritin tertiles (with lowest tertile as reference) at each stage of follow-up and patterns and slope of ferritin across the follow-up with the variation in MetS Z score. For the patterns of repeated ferritin measurements,

low/moderate ferritin levels (tertiles 1 and 2) at both stages of an interval or at all of the stages of follow-up were used as reference. Associations between ferritin level at each stage and Z scores or SD units of MetS components and IR were evaluated by linear regression with ferritin in SD units. Before calculating Z scores (ferritin and MetS components) and performing the regression analysis, the variables with skewed distribution were transformed to approximate to normal distribution. Ferritin, SBP, DBP, and CRP values were log-transformed in girls and boys; WC and triglycerides, in girls; and HDL-C, in boys. In boys, WC was transformed as (1/square of WC values)*-1 and triglycerides as (1/square root of triglycerides values)*-1. There were few covariates available to adjust the associations and these were: BMI Z score, Tanner stage, and haemoglobin levels. For the cross-sectional associations at 16–17 years old, CRP was available and, therefore, used in the adjustment model. Adjustment for sex and age was not needed since the analyses were conducted by sex and at the specific age of follow-up. All analyses were performed using Stata Statistical Software: Release 14. College Station, Texas.

2.3 Results

The Spanish and Chilean paediatric cohorts included in this chapter were not comparable since the age ranges and design of the studies were very different. The Spanish children were 3–14 years old and, all of them were prepubertal, whereas the Chilean children were evaluated at specific ages of 5, 10, and 16–17 years and cardiovascular risk outcomes were measured at 16–17 years. Differences were also observed in ferritin levels and MetS score between the two cohorts. The Spanish children had higher ferritin levels [median (interquartile range) 36 (26–52) µg/L] than the Chilean children at all groups (5 [21.6(14.6–30.4) µg/L], 10 [26.5(20.4–35.7) µg/L], and 16–17 years [26.3(15.7–30.4) µg/L]. In contrast, the Chilean children had a higher MetS score [median (interquartile range) -0.05 (-0.37 to 0.35) µg/L] vs. 0.008(-0.4 to 0.38)]. This difference in cardiometabolic risk was expected and can be attributed to the difference in age and sexual development.

2.3.1 Findings in the Spanish cohort

A comparison between included and excluded children revealed modest differences in the study variables. Initially, the included children were older and had higher values of BMI Z score, WC, SBP, and GGT and lower HDL-C levels than the excluded children (Appendix Table 2). However, most of these differences appeared to be attributed to the difference in age, since only the difference in BMI and SBP remained significant after adjustment for age (Appendix Table 2). Among the included children, at baseline, those with follow-up data were younger and had lower values of the iron markers and the rest of the study variables, except HDL-C and HbA1C levels, than those without follow-up data (Appendix Table 3). After adjusting for age, the above differences persisted for BMI Z score, iron markers, insulin and HOMA-IR, WC, and blood pressure (Appendix Table 3).

In the whole group, the median and interquartile ranges for ferritin and transferrin were 36(26–52) μg/L and 274(251–298) mg/dL. The characteristics of the study population are presented by sex in Table 1. At baseline, boys were significantly older and had higher levels of glucose, and GGT than girls. Girls had higher values of DBP, triglycerides, CRP, and insulin than boys. In the subgroup of those with follow-up data, boys had higher levels of HDL-C and HbA1C, and girls had higher values of insulin and triglycerides than the opposite sex. There were no significant differences by sex in the anthropometric variables at baseline and after follow-up. Differences by sex for MetS score were not calculated, as the scores were sex-specific. Ferritin (log-transformed) and transferrin were negatively correlated ($r = -0.207$, $P < 0.001$).

Table 1 Description of the Spanish cohort at baseline and outcome variables after follow-up (sub-sample)

	Baseline			After follow-up		
	Girls (n=350)	Boys (n=376)	*P value*	Girls (n=90)	Boys (n=83)	*P value*
Age (years)	7.7 (6.3-9.5)	8.1(6.6-10.1)	**0.038**	10.6(9.9-11.5)	10.8(9.7-11.7)	0.614
Cardio metabolic risk variables						
Waist (cm)	62.0(54.0-73.0)	64.2(53-76.7)	0.103	67(61-80)	67(60-77)	0.623
SBP (mmHg)	106 (99-114)	107(99-114)	0.925	105(96-111)	102(95-110)	0.427
DBP (mmHg)	61(56-66.2)	59.5(54-65)	**0.015**	58(52-62.2)	57.5(52-62)	0.637
Glucose (mg/dL)	86(82-91)	88(83-92)	**0.003**	87(82-91)	88(84-92.7)	0.163
TG (mg/dL)	55(45-75)	53(41-72.7)	**0.011**	58(46-73)	49(40-60)	**<0.001**
HDL-C (mg/dL)	54(46-62.3)	55.8(47.5-65)	**0.170**	54.5(47-62.2)	62(53-70)	**0.002**
Insulin (ulU/ml)	4.6(2.0-8.6)	3.7(1.0-7.2)	**0.004**	5.0(2.7-8.9)	3.9(1.6-6.5)	**0.030**
HOMA-IR	0.98(0.40-1.89)	0.80(0.21-1.63)	**0.011**	1.08(0.56-1.89)	0.80(0.33-1.45)	0.051
HbA1c(%)	5.3(5.1-5.5)	5.3(5.2-5.5)	0.215	5.2(5.0-5.5)	5.4(5.2-5.5)	**0.007**
MetS score	-0.02(-0.37 to 0.38)	0.03(-0.43 to 0.39)		-0.03(-0.39 to 0.33)	-0.15(-0.53 to 0.28)	
Iron markers						
Ferritin (µg/L)	35.9(25.8-53)	36(25-50)	0.642			
Transferrin (mg/dL)	271(249-296)	276.5(253.2-302)	0.129			
Covariates						
Height (cm)	130(120.1-141)	133(122-143.8)	**0.033**			
BMI Z score	0.53(-0.53 to 1.66)	0.51(-0.57 to 1.76)	0.890			
CRP (mg/L)	0.90(0.30-2.42)	0.60(0.22-2.20)	**0.005**			
ALT(U/L)	17(14-20)	18(14-21)	0.068			
GGT(U/L)	12(11-15)	13(11-15)	**0.040**			

Data are median (interquartile range). SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol. MetS, Metabolic syndrome.HbA1C, glycosylated haemoglobin. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein. ALT, Alanine aminotransferase. GGT, gamma-glutamyl transferase.

The prevalence of underweight and overweight/obesity was 11.0% and 39.8%, respectively. Anaemia was found in 26 (3.6%) children. Only two children had iron deficiency with ferritin levels <7 μg/L and 13 had ferritin levels <10 μg/L. Among 482 children, whose serum albumin was measured, none had low values of albumin (<3.2 g/dL), an indicator of under nutrition. At baseline, all the children were pre-pubertal.

Table 2 Cross-sectional and prospective relationships between age and sex-specific standardized values of iron markers at baseline and MetS and glucose metabolism markers

	Ferritin SD units at baseline (μg/L)			
	Model 1		Model 2	
	βeta (95% CI)	*P*	βeta (95% CI)	*P*
MetS Z score at baseline	**0.07 (0.02 to 0.11)**	**0.001**	0.01 (-0.02 to 0.04)	0.499
Change in MetS Z score per year of follow-up	**-0.02(-0.04 to -0.007)**	**0.008**	**-0.02(-0.04 to 0.004)**	**0.017**
	Transferrin SD units at baseline (mg/dL)			
MetS Z score	0.05 (0.01 to 0.10)	0.009	0.002 (-0.03 to 0.03)	0.887
Change in MetS Z score per year of follow-up	0.01(-0.003 to 0.04)	0.098	0.01 (-0.007 to 0.03)	0.179

Model 1: adjusted for age and sex. Model 2: adjusted for model 1 plus CRP, ALT and GGT levels and BMI Z score. For the prospective association, the above models also included MetS Z score at baseline as covariate. Significant associations are shown in bold.

Iron markers and MetS

At baseline, there were positive age/sex-adjusted cross-sectional associations of ferritin and transferrin Z scores with the MetS Z score, which were attenuated after further adjustments (Table 2). In sharp contrast, the prospective analysis showed an inverse association between the ferritin Z score and the change in MetS score per year of follow-up that was independent of covariates (Table 2). For every SD unit increase in ferritin, there was a decrease of 0.02 SD units in the change of MetS score. Transferrin levels were not longitudinally associated with the change in MetS score (Table 2).

In the analyses by sex, the above prospective inverse association between ferritin and change in MetS, described in the whole sample using the iron markers and the change in MetS Z score, was generally present in both girls and boys (Table 3). No additional trends or significant associations by sex were observed.

Table 3 Cross-sectional and prospective relationships between age and sex-specific standardized values of iron markers at baseline and MetS and glucose metabolism markers

Girls				
	Ferritin SD units at baseline (µg/L)			
	Model 1		Model 2	
	βeta (95% CI)	*P*	βeta (95% CI)	*P*
MetS Z score at baseline	**0.10 (0.04 to 0.16)**	**0.001**	0.04 (-0.007 to 0.08)	0.098
Change in MetS Z score per year of follow-up	-0.02 (-0.05 to 0.001)	0.061	-0.02 (-0.05 to 0.004)	0.099
	Transferrin SD units at baseline (mg/dL)			
MetS Z score	0.02 (-0.03 to 0.09)	0.342	0.002 (-0.04 to 0.04)	0.909
Change in MetS Z score per year of follow-up	0.02 (-0.01 to 0.05)	0.204	0.01 (-0.01 to 0.04)	0.334
Boys				
	Ferritin SD units at baseline (µg/L)			
	βeta (95% CI)	*P*	βeta (95% CI)	*P*
MetS Z score at baseline	0.04 (-0.02 to 0.10)	0.210	-0.01 (-0.06 to 0.03)	0.599
Change in MetS Z score per year of follow-up	-0.03 (-0.06 to 0.001)	0.059	-0.03 (-0.06 to 0.003)	0.075
	Transferrin SD units at baseline (mg/dL)			
MetS Z score	**0.08 (0.02 to 0.14)**	**0.008**	-0.001 (-0.04 to 0.04)	0.946
Change in MetS Z score per year of follow-up	0.01 (-0001 to 0.04)	0.294	0.01 (-0.02 to 0.04)	0.441
Model 1: adjusted for age and sex. Model 2: adjusted for model 1 plus CRP, ALT and GGT levels and BMI Z score. For the prospective association, the above models also included MetS Z score at baseline as covariate. Significant associations are shown in bold.				

Additional associations between iron markers and MetS components, HbA1C, and insulin resistance

In the cross-sectional analysis of the whole sample, ferritin Z score at baseline was inversely associated with the Z scores of fasting glucose and HbA1C, and no statistically significant prospective associations were observed between ferritin and the change over time in MetS-related variables, HbA1C and IR (Appendix Table 4). Transferrin Z score was positively associated with HDL-C Z score and the fall in the Z score of this marker over time (Appendix Table 4). There were age and sex-adjusted cross-sectional positive associations between ferritin and SBP, DBP, triglycerides, HDL-C levels, and HOMA-IR in crude analyses that did not remain statistically significant after adjustments for inflammatory, hepatic injury, and body mass markers.

The above cross-sectional associations in the whole sample between ferritin and fasting glucose Z scores and between transferrin and HDL-Z scores were observed in boys but not girls (Appendix Table 5). The prospective inverse association described for the whole sample between transferrin Z score and the change in HDL-C per year of follow-up was found only in girls (Appendix Table 5). There were also two sex-specific associations not seen in the whole sample; in boys, the ferritin Z score was positively associated with the change in HDL-C Z score over time, and in girls, this iron marker was also inversely associated with the fall in the triglyceride Z score over time (Appendix Table 5). However, in addition to simply identifying a statistically significant/ non-significant finding it is important to take into account the finding of wide confidence intervals with Beta coefficient values close to zero in some of the above associations (e.g. adjusted ferritin-glucose and transferrin-HDL-C in boys, Appendix Table 5) These suggest the findings are close to being consistent with the null hypothesis. Low statistical power contributes to these patterns and makes it difficult to establish whether any differences are clinically significant.

Removal of age, and baseline values of outcomes (in the longitudinal analyses), did not substantially affect the effect estimates and statistical significance of the

associations described above. Additional adjustments for anaemia or haemoglobin levels did not affect the relationships described previously.

2.3.2 Findings in the Chilean cohort

On comparing the distribution of study variables between included and excluded children at the different time points of evaluation (5, 10, 16–17 years old) there were minimal differences (Appendix Table 6). Haemoglobin levels were slightly higher in the 16–17-year-olds included, and the BMI was higher in the 10-year-olds in comparison with the excluded of the same age groups (Appendix Table 6). Also, among those included, there was a higher proportion of children assigned to receive high iron supplementation when they were infants (6–12 months of life) (Appendix Table 6).

The study variables are described by sex and age at follow-up in Table 4. No differences by sex were found at 5 years of age for ferritin (μg/L), haemoglobin, and BMI Z score. At 10 years of age, girls had higher sexual development than boys (Table 4). At adolescent stage, boys had higher levels of ferritin, haemoglobin, SBP and DBP, and glucose and girls had higher sexual development and HDL-C and insulin levels (Table 4). Lower ferritin levels in girls were expected because of menstruation, which typically begins at Tanner stage IV. The slope for average annual change in ferritin across the follow-up was higher in boys than girls [mean(SD) 0.67 (1.82) v. -0.50(1.38), $P < 0.001$].

Prevalence of underweight was 3% at 5 years, 5% at 10 years, and 7 % at adolescence. Prevalence of overweight/obesity was, in general, similar to that found in the Spanish cohort: 44.1%, 44.4%, and 34% at 5 years, 10 years, and at adolescence, respectively. None of the children had anaemia when they were 5 and 10 years old, and at adolescence, 17 (3%) had anaemia.

Table 4 Description of study variable in the Chilean cohort

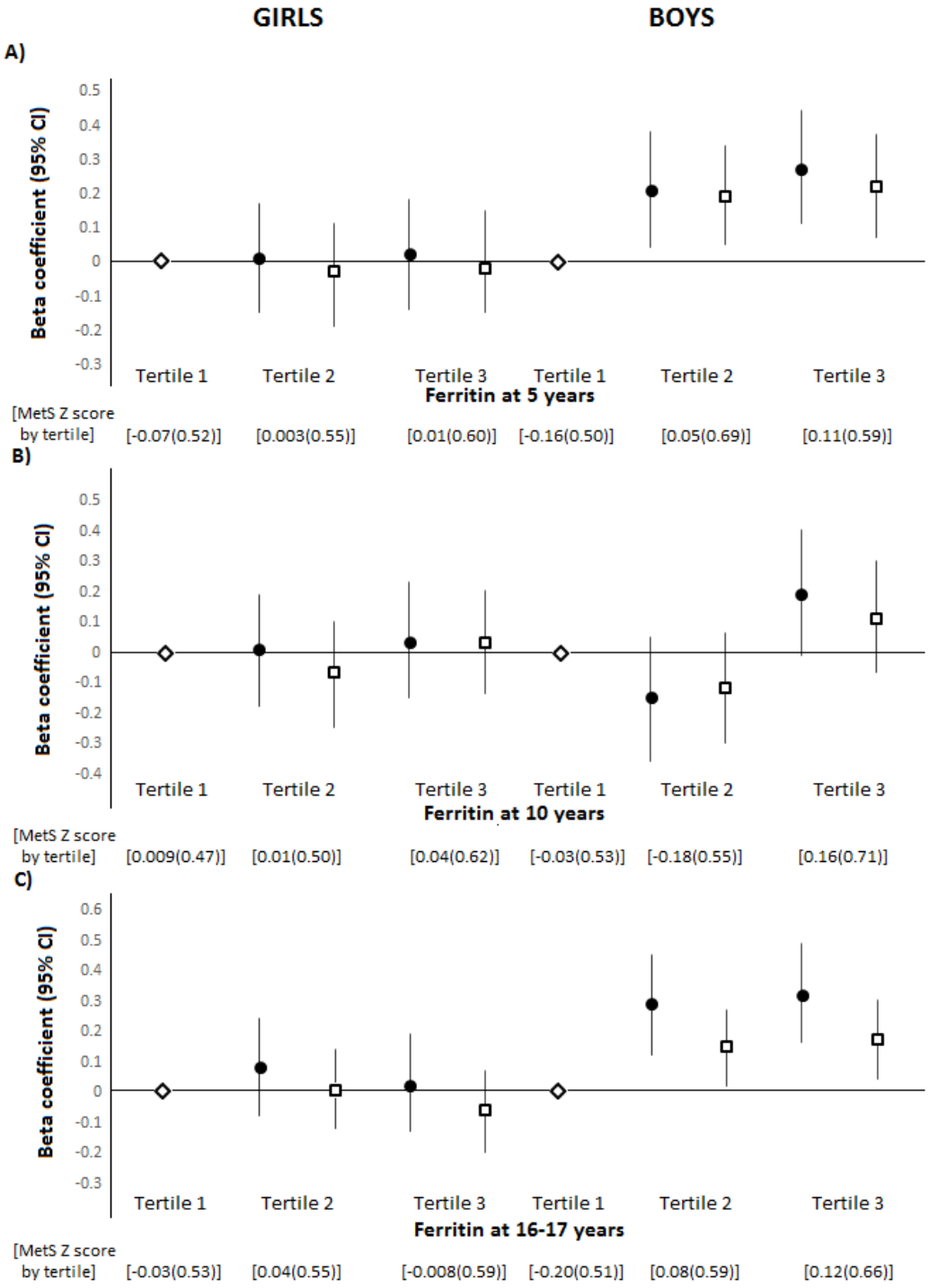
		Girls	Boys	*P value*
At 16-17 years		***n*=270**	***n*=297**	
Ferritin (µg/L)		19.1(11.7-28.2)	32.7(22.7-7.1)	**<0.0001**
Haemoglobin (g/dL)		13.7(13-14.3)	15.9(15-16.6)	**<0.0001**
CRP (mg/L)		0.45(0.15-1.35)	0.38(0.12-1.20)	0.2849
BMI Z score		0.67(0.03 to 1.26)	0.42(-0.22 to 0.42)	0.0813
Tanner stage (n(%))				
	2	0.0%	0.6	**<0.0001**
	3	5.9	16.4	
	4	53.3	56.9	
	5	40.7	25.9	
Waist (cm)		79.6(72.5-88.3)	78.4(73.5-86.4)	0.7939
SBP (mmHg)		108(100-113.5)	115(108-122)	**<0.0001**
DBP (mmHg)		68(61-71)	70(65-75)	**<0.0001**
Glucose (mg/dL)		86.1(80.9-92.1)	90.7(84.4-96.7)	**<0.0001**
TG (mg/dL)		74.9(57.9)	73.7(56.1-102.2)	0.9309
HDL-C (mg/dL)		41.5(35.1-49.5)	36.6(30.8-42.2)	**<0.0001**
Insulin (ulU/ml)		7.1(5.1-10.3)	6.2(4.3-9.7)	**0.0399**
HOMA-IR		1.48(1.05- 2.15)	1.40(0.96-2.17)	0.3315
MetS score*		-0.01(-0.36 to 0.33)	-0.07(-0.38 to 0.38)	
At 5 years		***n*=268**	***n*=297**	
Ferritin (µg/L)		22.3(15.3-32.3)	21.3(14.3-29.1)	0.0954
Haemoglobin (g/dL)		12.9(12.5-13.4)	12.9(12.4-13.5)	0.9584
BMI Z score		0.87(0.23-1.57)	0.83(0.18-1.55)	0.8449
At 10 years		***n*=183**	***n*=198**	
Ferritin (µg/L)		27.4(21.1-36.8)	25.6(19.8-34.9)	0.5094
Haemoglobin (g/dL)		13.6(13.0-14.1)	13.7(13.1-14.2)	0.4204
BMI Z score		0.87(0.01-1.42)	0.94(0.26-1.56)	0.0900
Tanner stage				
	1	39.3	70.7	**<0.0001**
	2	43.1	29.2	
	3	16.9	---	
	4	0.5	---	

***Difference by sex does not apply since MetS Z scores were calculated as sex specific. SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein.**

Ferritin levels in the highest tertile at five years (compared to the lowest tertile) were positively associated with MetS risk Z scores in adolescence (age 16-17) in boys (Figure 2), and this association was unaffected by adjustment for baseline BMI Z scores and haemoglobin levels. Also, in boys, at adolescence, there was a significant

cross-sectional association between ferritin levels in the highest tertile and increasing MetS risk Z score, independent of adjustment for BMI Z score, CRP levels, haemoglobin levels, and Tanner stage (Figure 2). No significant associations were found between ferritin levels at 10 years and MetS Z scores at adolescence (age 16-17). In girls, no significant associations were found between ferritin levels at any age and MetS Z scores in adolescence (Figure 2).

Figure 2 Linear regression for longitudinal (A and B) and cross-sectional (C) associations between tertiles of ferritin at the three points of follow-up and the MetS Z score at adolescence.



A, n= 565. B= n=381. C, n=567. ◇ Reference. ● Unadjusted. □ Adjusted: Longitudinal associations with ferritin at 5 and 10 years were adjusted for the respective baseline BMI Z score and haemoglobin level, and tanner stages for 10 year's evaluation; Cross-sectional association at 16-17 years were adjusted for BMI Z score, CRP level, haemoglobin level and tanner stage. [Means (SD)] of MetS Z score by tertiles are also provided.

The above analysis was replicated by using only the sample with no missing values for the main variables and covariates for all the three points of follow-up (n = 379). The associations with MetS remained similar: in girls at 5, 10, and 16–17 years, the adjusted beta coefficients (highest vs. lowest tertile of ferritin) were -0.03(-0.21 to 0.14) P=0.689, 0.05(-0.12 to 0.24) P=0.524, and 0.12(-0.05 to 0.31) P=0.172,respectively); in boys, the adjusted beta coefficients were 0.23(0.04 to 0.42, P=0.015), 0.10(-0.09 to 0.29, P =0.297), and 0.35(0.16 to 0.54) P < 0.001), respectively.

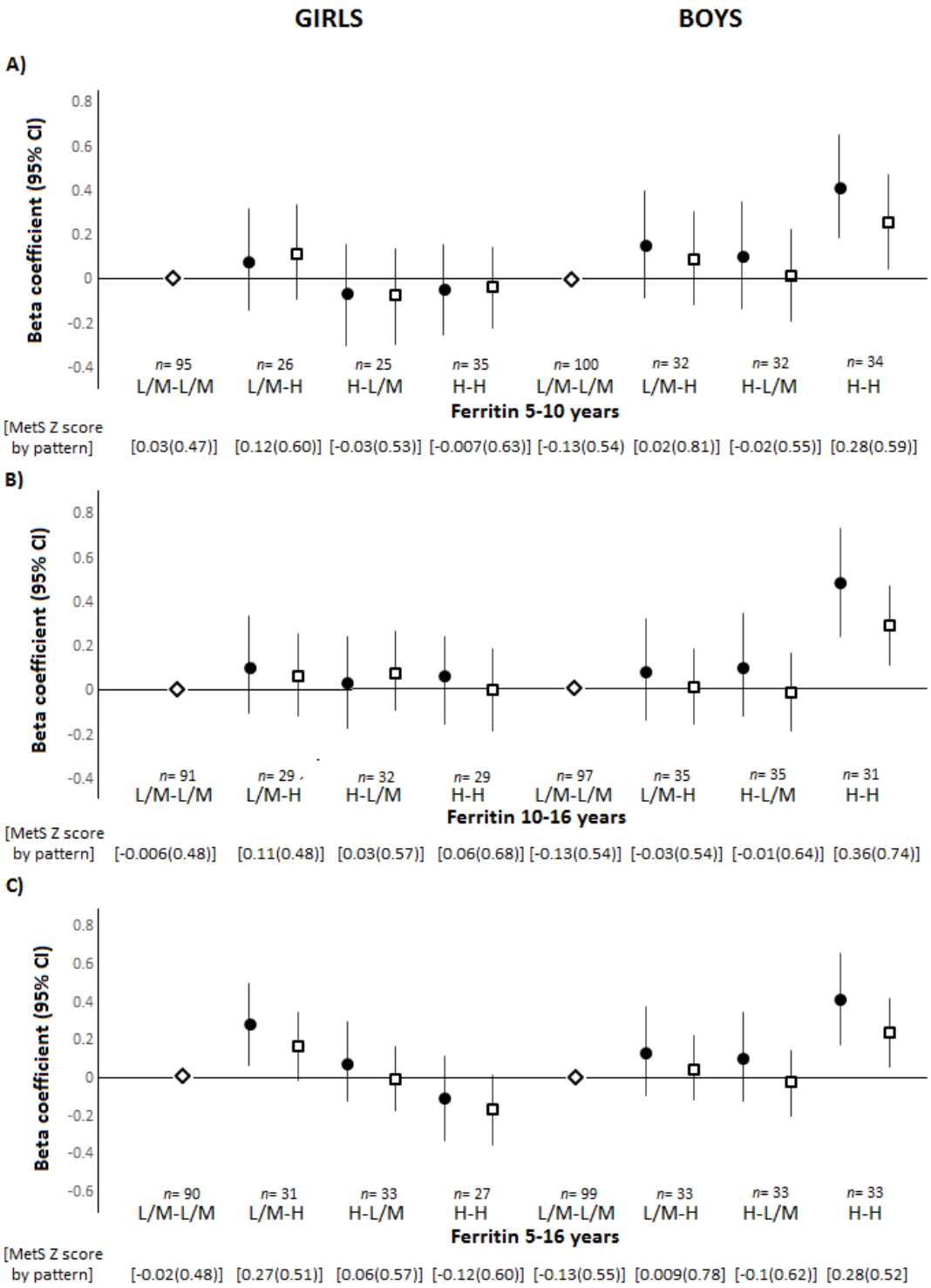
Having high, compared to low/moderate, ferritin levels at two or more time periods between 5 and 16 years was related to higher MetS risk Z score at age 16-17 in boys only (P < 0.05) (Figure 3). A sensitivity analysis by adjusting the associations in Figure 3 for baseline values of covariates along with changes in these across the intervals, showed very similar beta coefficients to those reported in Figure 2 (Appendix Table 7).

The presence of high ferritin levels at the three time points was positively associated with MetS risk Z score in boys [beta(95%CI) = 0.31(0.11 to 0.52)] but not in girls (Appendix Table 8). However, the number of children included in some cells was very small (Appendix Table 8).

The effect of having persistently high ferritin levels at 10 and 16 years or at all three ages had a slightly stronger association with MetS Z scores in boys (beta coefficients 0.30 and 0.31, respectively) than for the association at single points mentioned previously in Figure 3 [beta coefficients = 0.23(5 yr), 0.11(10 yr), 0.21(16–17 yr)].

The slope values for ferritin levels per year, as continuous variables, were marginally and positively associated with higher MetS risk Z score in girls but not in boys. When comparing highest and lowest tertiles of slope, the association with MetS risk Z score in girls became stronger and statistically significant (Table 5).

Figure 3 Linear regressions associations between MetS Z score at 16/17 years and patterns of combined high (H) and low/moderate (L/M) ferritin concentration throughout the follow-up.



◇ Reference. ● Unadjusted. ◘ Adjusted: associations were adjusted for the values of BMI Z score and haemoglobin level and tanner stage(if applicable) at the end of the interval (e.g interval 5-10 years included covariates of BMI Z score, haemoglobin levels, and tanner stage at 10 years old). For major

comparability of the intervals of follow-up, this analysis used cases with no missing values for all of the three points of measurements (n=379 at each stage).

Table 5 Beta coefficients for MetS Z score by slope of ferritin concentration across the three stages of follow-up

	Girls			
	Unadjusted		Adjusted*	
	Beta (95% CI)	P value	Beta (95% CI)	P value
Slope of ferritin (continuous)	**0.06 (0.005 to 0.11)**	**0.031**	*0.04 (-0.002 to 0.09)*	*0.062*
Slope of ferritin				
Tertile 1	0(Reference)		0(Reference)	
Tertile 2	0.07 (-0.10 to 0.26)	0.406	0.09 (-0.06 to 0.25)	0.255
Tertile 3	**0.25 (0.06 to 0.44)**	**0.008**	**0.21 (0.05 to 0.38)**	**0.007**
	Boys			
	Unadjusted		Adjusted*	
	Beta (95% CI)	P value	Beta (95% CI)	P value
Slope of ferritin (continuous)	0.01 (-0.03 to 0.05)	0.671	0.01 (-0.02 to 0.04)	0.471
Slope of ferritin				
Tertile 1	0(Reference)		0(Reference)	
Tertile 2	-0.01 (-0.23 to 0.19)	0.870	0.01 (-0.13 to 0.17)	0.823
Tertile 3	0.08 (-0.13 to 0.29)	0.450	0.10 (-0.04 to 0.26)	0.179

Appendix Table 9 shows the results of linear regression analyses for relationships between sex-specific Z score of ferritin level at the three points of follow-up and sex-specific Z scores for MetS components and IR at adolescence. In girls, the Z scores for ferritin levels at 10 and 16–17 years of age were significantly (P = 0.046 and P = 0.014, respectively) and independently associated with the Z score of IR but not with any of the MetS components at the adolescent stage. In boys, ferritin level Z score at 5 years old was inversely associated with the Z score of HDL-C in adolescence in unadjusted and adjusted models, whereas significant unadjusted associations with Z scores of WC, HDL-C and IR did not remain significant after adjustment for covariates. In adolescence, there were significant cross-sectional associations between Z scores of ferritin and fasting glucose, triglycerides, and IR independent of adjustment for covariates in boys only.

Further adjustment for trial groups of iron supplementation at infancy did not affect the associations previously described. A similar pattern was observed when

haemoglobin levels were removed from the adjustment models. There were no significant findings on analysing female and male sexes together ($P > 0.05$), presumably owing to the marked differences by sex in the outcomes at adolescence.

2.4 Discussion

The ferritin-cardiometabolic risk relationship was different in the two paediatric cohorts. While serum ferritin was inversely associated with cardiometabolic risk, fasting glucose, and glycosylated haemoglobin levels in the Spanish children, iron stores at prepubertal age were positively associated with cardiometabolic risk in Chilean adolescents. Moreover, in this latter cohort, patterns of sustained high levels of ferritin throughout childhood were associated with adverse metabolic profile in adolescence. In the Chilean girls, linear increases in ferritin, in terms of the slope of ferritin levels from measurements at 5, 10 and 16 years, were independently associated with MetS risk. The above findings highlight iron metabolism as a factor associated with cardiometabolic risk from early life and suggest that there may be differences in the association by sex and age.

2.4.1 Contrast with previous studies

Only five studies conducted to date on the association of ferritin and other iron status markers with cardiometabolic risk variables in children were identified from a systematic literature search performed in May 2016. All studies use cross-sectional designs and four involved Asian paediatric populations (Table 6). All these studies included children and/or adolescents from general populations, except the study by Bougle and Brouard of overweight and obese Canadian children (100). This latter study is the only one, along with the present studies, that reported associations adjusted for inflammation markers. Lee et al., similar to our study, evaluated outcomes of MetS and its components (101) but with adjustments for age and sex only. The other studies evaluated MetS components (categorical or continuous variables). In general, the studies reported higher ferritin values than those reported in the Chilean and the Spanish cohorts, showed no significant association between ferritin and most of the cardiometabolic risk outcomes, and did not include robust adjustments (Table 6). Unadjusted and adjusted associations tended to be consistent

in the studies reviewed. Zhu et al. did not find an association between ferritin and any MetS component in 1126 Chinese children (102). Ferritin levels were significantly and positively associated with WC in the studies by Lee et al. and Jeon et al. involving Korean children (101, 103) and with triglycerides in the studies by Bougle and Brouard and Kim et al. (100, 104), and were inversely associated with HDL-C in the studies by Bougle and Brouard, Kim et al., and Lee et al. (100, 101, 104). None of the studies reported associations between ferritin and fasting glucose or blood pressure.

Although ferritin was, in general, not associated with the MetS components in the studies by Lee et al. and Zhu et al., there were a few contrasting associations with sTfR. While Lee et al. described unadjusted higher levels of sTfR in children with MetS (101), Zhu et al. found an inverse association between sTfR and high triglycerides and low HDL-C with and without adjustments for age, gender, physical activities, and dietary behaviours (102). These latter authors also reported an inverse association between transferrin and low HDL-C. The findings by Zhu et al. are coherent with the iron excess-cardiometabolic risk pattern, since lower levels of sTfR and transferrin represent high iron status. It is unclear if the positive association of sTfR-MetS in the study of Lee et al., which reflected lower iron status for MetS, would be independent of the confounding factors.

The contrasting and significant cross-sectional findings in Chilean boys with regard to the above studies could be explained in terms of differences in age range and study design. In the Chilean cohort, participants were evaluated at the same age at each evaluation and cardiometabolic risk outcomes were observed at the adolescent stage (16–17 years), a higher and narrower age range than in the above studies.

Table 6 Studies in children on ferritin and cardiometabolic risk*

Authors, year (reference)	Study	Location/ Universe	Study/ Year of survey	Age range (years)	Male (%)	Total sample	Cardiovascular risk markers (outcomes and analysis-effect estimate)	Ferritin : Variable approach (Continuous /categorical-ordinal) Average levels	Association		Adjustments
									Yes	No	
Lee et al., 2014 (101)	Cross-sectional	Korea/ school-aged children	Korean Pediatric Cohort Study	7 and 10	50.5	1350	1-Metabolic syndrome (categorical) [difference in ferritin levels by having or not MetS] 2-Glucose, WC, HDL-C, SBP, DBP, HOMA-IR and TG (continuous). [Partial Pearson Correlations]	Continuous Mean (SD) 36.8 (19.5)	With WC (r=0.07) and HDL-C (r=-0.10) [p<0.05].	With MetS. Glucose (r=-.004) HOMA-IR (r=0.03) TG (r=0.03) SBP(r=0.03) DBP(r=0.04) [p>0.05].	Age and sex (for MetS components) Unadjusted analysis for MetS.
Kim et al., 2016 (104)	Cross-sectional	Korea/ Paediatric general population	Korean National Health and Nutrition Survey IV 2009-2010	10-18	64.1	1879	HDL-C, TG, and HOMA-IR (continuous). Sex-stratified multivariable linear regression.	Continuous Median(IR) Girls 26.9 (25.4-28.6) µg/L Boys 42.7 (40.7-44.9) µg/L	With HDL-C (inverse) in both sexes. With TG in boys only.	With HOMA-IR in both sexes.	Age, height, weight, place, smoking status, drinking, physical activity, serum 25(OH)D level, total daily energy intake, fat intake, protein intake, calcium intake, and menarche in girls

Authors, year (reference)	Study	Location/ Universe	Study/ Year of survey	Age range (years)	Male (%)	Total sample	Cardiovascular risk markers (outcomes and analysis-effect estimate)	Ferritin : Variable approach (Continuous /categorical-ordinal) Average levels	Association		Adjustments
									Yes	No	
Jeon et al., 2013 (103)	Cross-sectional	Korea/ Paediatric general population	Korean National Health and Nutrition Survey V 2010	10-18	54.4	849	High glucose as ≥ 100 mg/dL High TG as ≥ 100 mg/dL Low HDL-C as ≤ 40 mg/dL High blood pressure (SBP≥130 or DBP≥85 (mmHg)) High WC (WC≥90th). [Linear regression with ferritin as outcome]	Continuous Mean (SD) Girls 30.6 (1.3) µg/L Boys 50.5 (2.3) µg/L	With high WC only	With High glucose , high TG , low HDL-C, and high blood pressure (SBP≥130 or DBP≥85 (mmHg))	Age
Bougle and Brouard et al.,2012 (100)	Cross-sectional	France/ overweight and obese children attending specialized outpatient clinic	Research on child obesity/ NP	11.4 ± 3.0 years old	53	502	Glucose, SBP, DBP, HDL-C, TG, and HOMA-IR (continuous). Multivariable linear regression.	Continuous Mean (SD) 41.7 (22.9) µg/L	With HOMA-IR , and TG and HDL-C (inverse).	Glucose, SBP, DBP, and HOMA-IR	Age, sex, BMI Z score and fibrinogen levels.
Zhu et al., 2016 (102)	Cross-sectional	China/ school-aged children	Epidemiological study in Guangzhou, China/20	7-14	46.5	1126	High glucose as ≥ 5.6 mmol/L High TG as ≥ 1.7 mmol/L Low HDL-C as < 1.03 mmol/L	Categorical (quartiles , and first/lowest quartile as reference) Median(IR)	None of the MetS components	With all of the MetS components	Age, gender, physical activities, and dietary behaviours

Authors, year (reference)	Study	Location/ Universe	Study/ Year of survey	Age range (years)	Male (%)	Total sample	Cardiovascular risk markers (outcomes and analysis-effect estimate)	Ferritin : Variable approach (Continuous /categorical-ordinal) Average levels	Association		Adjustments
									Yes	No	
			14				High SBP ($\geq 90^{th}$) High DBP ($\geq 90^{th}$) High WC ($\geq 90^{th}$). [Logistic regression with ferritin as independent variable]	56 (41-75) µg/L			
Present study	Cross-sectional/ Longitudinal	Chile/General paediatric population	Epidemiological study in Santiago, Chile	5, 10 and 16-17 years	52.3	567	1-Metabolic syndrome at 16-17 years (continuous) 2-Glucose, WC, HDL-C, SBP, DBP, HOMA-IR and TG at 16-17 years (continuous). Sex-stratified multivariable linear regression.	Continuous Categorical at 5, 10 and 16-17 years (tertiles , and first/lowest tertile as reference) Median(IR) 5 years 21.6(14.6-30.4) µg/L 10 years 26.5(20.4-35.7) µg/L 16-17 years 26.3(15.7-30.4) µg/L	Girls: Association of ferritin at 10 and 16 years with HOMA-IR and MetS Boys: Association (inverse) of ferritin at 5 years with HDL-C. Cross-sectional Association of ferritin with glucose, TG, HOMA-IR and MetS		Associations with ferritin at 5 and 10 years adjusted for BMI Z score, haemoglobin level and tanner stage Associations with ferritin at 16-17 years adjusted for BMI Z score, CRP level, haemoglobin level and tanner stage

Authors, year (reference)	Study	Location/ Universe	Study/ Year of survey	Age range (years)	Male (%)	Total sample	Cardiovascular risk markers (outcomes and analysis-effect estimate)	Ferritin : Variable approach (Continuous /categorical-ordinal) Average levels	Association		Adjustments
									Yes	No	
Present study	Cross-sectional/ Longitudinal	Spain/ General paediatric population	Epidemiological study in Girona, Spain	3-14 years	51.8	726	1-Metabolic syndrome score at baseline and after 3.7 ± 0.8 years (continuous) 2-Glucose, WC, HDL-C, SBP, DBP, TG, HOMA-IR and HbA1C at 16-17 years (continuous). Sex-stratified multivariable linear regression.	Continuous 36 (26-52) µg/L	Cross-sectional inverse associations with HbA1C and fasting glucose Longitudinal inverse association with the change in MetS score		Age, sex, CRP, ALT and GGT levels and BMI Z score. For the prospective association, the above models also included MetS Z score at baseline as covariate.

*** found in PUBMED by applying the searching: iron AND (cardiometabolic risk OR cardiovascular risk OR metabolic syndrome) AND (children OR adolescents) (until May 25/2016). [in vitro studies, genetic studies and those conducted adults populations or patients with diseases were not included]. WC, waist circumference. LDL-C, LDL cholesterol. HDL-C, HDL cholesterol. TG, triglycerides. DBP, diastolic blood pressure. SBP, systolic blood pressure. FG, fasting glucose. Q, quartile.**

When contrasting results from the Spanish cohort with previous findings from studies in children, including those from the Chilean cohort, it is important to take into account some differences among the studies. Although the Spanish cohort had a similarly wide age range as the studies cited in Table 6, I applied more robust adjustment for potential confounding factors. Several positive cross-sectional associations between ferritin and MetS components were lost after adjusting for covariates, with a major attenuating effect by transaminases and BMI. The positive associations found in the Chilean cohort might have been attenuated if it had been possible to adjust for markers of hepatic function.

Longitudinal association of ferritin-MetS and discrepancy between the Chilean and Spanish cohorts

The longitudinal and cross-sectional positive associations between ferritin at each stage of childhood (except at 10 years) and MetS observed in the Chilean cohort were significant in boys but not in girls. This sex difference may be explained by a threshold effect derived from higher levels of cardiometabolic risk factors in boys at the adolescent stage, rather than by higher iron stores in boys since at 5 years, there was no significant sex difference in ferritin levels. However, there are not a consistent findings about differences by sex in the ferritin-MetS association in adults. There are studies showing either stronger or sex-specific significant association in men (7, 105) or women (pre and postmenopausal) (106-108) or significant associations in both sexes (22). All studies consistently described higher MetS prevalence or incidence in postmenopausal women and men than in premenopausal women, and higher ferritin levels in men than in pre- and postmenopausal women.

In contrast, low iron status (and not iron excess) was prospectively associated with a higher MetS risk score in the Spanish children. Some previous epidemiological studies found increased cardiometabolic risk in subjects with low iron status. Inconsistent direction and patterns of associations have been found between iron markers (iron intake, transferrin saturation, and serum ferritin concentration) and risk of CHD in longitudinal studies in adults (28). Nutritional status is an important potential confounding factor, at least in some populations. There is increasing

evidence describing the double burden of malnutrition and cardiometabolic risk, particularly in developing countries. Coexistence of at least one nutritional deficiency (iron or vitamin A) and one cardiometabolic risk factor (among overweight/obesity, abdominal obesity, hypertension, hyperglycaemia, diabetes, or dyslipidaemia) was observed in 23.5% of a West-African population, and this phenomenon was significantly higher in the low-income group (109). Interestingly, iron deficiency has been associated with elevation in fasting blood glucose despite increased insulin sensitivity in animal models (110). A dose-response relationship has been described between anaemia and hyperglycaemia in rats (31). However, mechanisms underlying this association are still unknown.

Along with the longitudinal association with MetS, there were another two inverse cross-sectional associations of serum ferritin with fasting glucose and HbA1C in the Spanish children, supporting the association between low iron status and cardiometabolic risk. HbA1C shows a spurious increase in the case of iron deficiency owing to an increase in the lifespan of erythrocytes. In the absence of concomitant rise of glucose levels in iron deficiency, an isolated finding of association between increased HbA1C and iron deficiency is spurious, as recently noted in a systematic review (111). Therefore, the inverse ferritin-HbA1C association described here does not appear to be entirely spurious.

The Spanish children did not show evidence of malnutrition on the basis of serum albumin (measured in 482 subjects) and no differences in weight or nutritional score were identified between children with low and high ferritin levels. Additional adjustment for anaemia did not affect the associations between iron status and cardiometabolic risk. The findings could be related to subclinical iron deficiency or life-course physiological transitions.

Subclinical iron deficiency, a possible explanation for the findings, is supported by a recent recommendation for higher cut-off points to establish iron deficiency in adults (serum ferritin <30 μg/L instead of <12 μg/L) (112), and normal ranges for children also need to be reviewed. However, against the above hypothesis is the fact that

Chilean children who showed a marked positive ferritin-cardiometabolic risk association had lower ferritin concentrations than the Spanish cohort. This observation along with the previous comparison of the existing few paediatric studies implies that the association between iron metabolism markers and cardiometabolic risk during infancy and childhood may differ by populations and geographical regions but not simply as a consequence of different levels of iron stores. In fact, the MetS score was higher in the Chilean children, and thus, a different distribution in cardiometabolic risk markers might explain the discrepancies in statistical significance and/or the direction observed in the two cohorts evaluated in this chapter.

With regard to the life-course physiological transition approach, the metabolic processes related to glucose homeostasis may be more iron-dependent in early stages of life than in adulthood. Changes in cardiometabolic risk markers could be sensitive to modest reductions in iron status within the narrow normal range suggested for children as opposed to for adults. Children do not accumulate as much iron as adults since their body compartments are still developing, and negative balance of iron storage is a more common finding than iron excess with physiological implications.

Pattern of repeated ferritin measurements and metabolic syndrome: Chilean cohort

The relationship between repeated measurements of ferritin and MetS appeared to differ by sex. In boys, a pattern of sustained high ferritin levels throughout the follow-up was significantly associated with MetS risk, but in girls, a linear change in ferritin levels across follow-up, but not the pattern of sustained high ferritin, was associated with MetS risk. This suggests that in girls, increases in ferritin across childhood, regardless of the starting point, are associated with adolescent cardiometabolic risk, and in boys, the association is markedly influenced by the threshold effects of ferritin and/or cardiometabolic risk outcomes. There are no studies on the trajectories of ferritin and cardiometabolic risk in adults although one study evaluated changes in ferritin with regard to development of MetS after 6.5 years' follow-up (113). In this study conducted in a Finnish population, men or

women who had incident MetS also had higher changes in ferritin values between baseline and the end of the follow-up (113). Further study of patterns of sustained high ferritin and slopes from repeated measurements of ferritin over time and their associations with cardiometabolic risk and outcomes in other paediatric populations to determine if the differences by sex in the present study are replicated.

Ferritin and insulin resistance

Ferritin was not associated with HOMA-IR in the Spanish cohort in cross-sectional or prospective analyses, and in the Chilean children this association was significant only in girls in terms of association with ferritin levels at 10 years of age. In addition, none of the three studies that investigated the relationship between ferritin and HOMA-IR in Table 6 found these variables significantly associated (100, 101, 104). This finding in Chilean girls is inconsistent with the lack of association between ferritin (at 10 and 16 years) and MetS score in the same sub-group. Given that the relationship between iron and glucose/insulin metabolism is bi-directional (9), insulin levels could potentially modulate iron stores throughout the growth process in girls. It is also plausible that the ferritin-insulin association is driven by an additional unknown factor not measured in this study, or it may also be a chance finding.

Transferrin, cardiometabolic risk and insulin resistance: Spanish cohort

The Spanish but not the Chilean cohort included the evaluation of an additional iron marker. Interestingly, although there was no evidence of a ferritin-IR association, transferrin was significantly associated with IR in the whole sample of the Spanish children. Transferrin levels were also positively associated with HbA1C, HDL-C, changes in SBP and DBP per year of follow-up, and with the fall in HDL-C levels over time. The direction of the above associations show the potential role of low iron status (the higher transferrin levels the lower the body iron), supporting the findings on ferritin and MetS in the Spanish cohort. However, ferritin and transferrin were individually associated with different cardiometabolic risk markers, making it difficult to conclude a consistent and solid pattern of low iron status-cardiometabolic risk. Among the paediatric studies in Table 6, only the one by Zhu et al. included serum transferrin as an additional iron marker. In this study of a Chinese general

paediatric population, there was only an isolated inverse association between transferrin and HDL-C levels in both the unadjusted model and model adjusted for age, sex, physical activity, and dietary habits (102). The direction of this association is opposite to that described in the Spanish children I have reported in this chapter. In the same study involving Chinese children, ferritin levels were not related to MetS-related variables although a positive association with total cholesterol was found. The Chinese and Spanish children had relatively comparable age ranges and HDL-C levels. However, iron status was lower in the Spanish cohort [transferrin mean (SD) 276 ± 36.4 vs. 240 ± 43 μg/dL; ferritin median (IQR) 36 (25–52) vs. 56(41–75)]) (Table 6), but it is still difficult to deduce the potential reason behind the discrepant findings between the two studies other than the role of chance. The inverse transferrin-HDL-C association described in the Chinese children might be not significant after further adjustment for BMI, transaminases, and CRP levels. On the other hand, the positive associations between transferrin, HOMA-IR, HDL-C, and blood pressure in the Spanish children are in line with cross-sectional and prospective associations reported in a French adult population (7).

In the study by Zhu et al., involving Chinese children, sTfR levels (which were not available for the cohorts described in this chapter), were inversely related to HDL-C and WC (102). This finding on sTfR associations is paradoxical, since the directions of the associations with HDL-C and WC would be expected to be opposite if there were a consistent link with cardiometabolic risk (higher WC, lower HDL-C). This observation supports the previously proposed idea of physiological transitions instead of true cardiometabolic risk involved in the relationships between iron markers and metabolic profile variables in school-aged children (5–12 years).

2.4.2 Limitations

Several limitations have to be mentioned here. In the case of the Chilean cohort analysis, first, because of unavailability of variables, there was no evaluation of cross-sectional associations between ferritin levels and cardiometabolic risk at 5 and 10 years since cardiometabolic risk outcomes were measured only at the adolescent stage. Second, there were no available measures of subclinical/clinical inflammation

at 5 and 10 years, meaning it is not possible to investigate the potential for ferritin acting as an acute phase reactant. However, in general, the children can be expected to have had good health status when they attended the appointments for blood samples. Adjustment for BMI Z score might partly have corrected the associations for the potential effect of adiposity-related subclinical inflammation on ferritin levels. It is also important to bear in mind that CRP levels in adolescence were not associated with cardiometabolic risk and IR and did not affect the associations in the multivariable models (data not shown). Third, unlike the Spanish cohort analysis, the associations reported in the Chilean children were not adjusted for hepatic function markers such as transaminases. Serum ferritin levels could be increased via the release of proteins from damaged hepatocytes although, again, it is suggested that liver damage is rare in children. With regard to the limitations of the study involving Spanish children, the follow-up was achieved only for almost a quarter of the whole sample, so the longitudinal findings for that cohort may not be generalisable and should be considered with caution since the power was limited. Similarly, several associations described in this Spanish cohort may still lack clinical relevance given their broad confidence intervals with values close to being consistent with a null effect and thus larger studies in prepubertal populations are required to establish whether a true effect exists. Unlike the case of the Chilean cohort, in the Spanish cohort, it was not possible to adjust for changes in sexual maturity since there were few cases with available information on Tanner stage after follow-up. In addition, the multiple statistical tests imply the likelihood of chance findings reported in this chapter.

2.4.3 Strengths

The two analyses in this chapter have some relevant strengths. To the best of my knowledge, these are the first studies evaluating longitudinal association between ferritin levels and cardiometabolic risk in paediatric populations. Moreover, the Chilean cohort analysis is the first study analysing patterns of three repeated measurements of ferritin over time regarding cardiometabolic risk. Both studies are the first to adjust the ferritin-cardiometabolic risk association in children for systemic inflammation markers, such as CRP levels. The evaluation of the children at specific

ages in the Chilean cohort allowed a more precise characterisation of the association in terms of a more homogeneous group of subjects than previous studies in children with wider age ranges. In the Spanish cohort, there was simultaneous evaluation of markers of iron storage and transport with regard to IR, MetS, and its components with a broad set of covariates for multivariate cross-sectional and longitudinal analyses.

2.5 Conclusions

The association between markers of iron status and cardiometabolic risk in children is complex and it appears that both low and high iron status may be related to cardiometabolic risk in different populations. It is uncertain if the association between high ferritin and higher cardiometabolic risk reported in the Chilean cohort, commonly described in adults, would remain significant after adjustments for hepatic function markers. Similarly, the association between low iron status and cardiometabolic risk reported in the Spanish cohort requires replication in larger samples.

Both serum ferritin levels at different time points in childhood and patterns of sustained high concentration throughout childhood were associated with MetS and IR but with differences by sex in the Chilean children. Patterns of sustained high ferritin and slopes from repeated measurements of ferritin over time should be tested for cardiometabolic outcomes in adults and other paediatric populations to establish whether the difference by sex in the present study is replicable.

The next chapter will evaluate similar associations to those presented here, in two populations of Scottish adults, along with a meta-analysis of observational studies on ferritin, MetS and its components.

Chapter 3

Cross-sectional studies in Scottish adult populations and a systematic review/meta-analysis of observational studies on serum ferritin, metabolic syndrome and its components

3.1 Introduction

Studies of the association between iron status, MetS, and its components in adult populations that have not previously been studied have the potential to add to existing knowledge. I did not find previous studies on the association between ferritin levels and MetS or its components in British populations. The Viking Health study performed in Shetland 2013–2015 includes measurements of ferritin and all the components of MetS. The nationally representative Scottish Health Surveys (SHeS) performed in 1995 and 1998 included measurements of ferritin and three out of the five MetS-related variables: WC, HDL-C, and blood pressure. In this chapter, I have used these two studies to investigate whether ferritin levels were independently associated with MetS and its components in these two Scottish populations.

Several systematic reviews and meta-analyses have described positive associations between ferritin and risk of T2D (19, 114-116), but there is limited evidence for the association between ferritin and MetS. One recent meta-analysis reported an overall positive association but did not investigate associations of ferritin with individual components of the MetS (18). Moreover, the role of important confounders such as BMI and hepatic function markers or threshold effects of ferritin levels in the associations reported in published studies is not always clear. Several recent studies on the topic have been published between 2014 and 2015 which have not been included in previous meta-analyses, justifying an updated review to address the gaps mentioned above with more statistical power. Therefore, I conducted a systematic review and meta-analysis of ferritin, MetS, and its individual components, adding the effect estimates from the cross-sectional studies of two Scottish populations.

3.2 Methods

3.2.1 Study 1: Viking Health study – Shetland and SHeS 1995–1998

Study population

The Viking Health Study – Shetland (VIKING) is a genetic epidemiology study on a relatively isolated population in the north of Scotland. For this study, 2,105 volunteers with at least two Shetlandic grandparents were examined over two years from March 2013 to March 2015. Each participant attended a measurement clinic and a venepuncture clinic to give a fasting blood sample at a dedicated research centre in Lerwick, Shetland Islands. The final sample consisted of 2,047 individuals after excluding 58 individuals with missing values for ferritin, MetS-related variables, insulin and glycosylated haemoglobin (HbA1C), or covariates. Ethical approval was issued by the Multi-centre Research Ethics Committee for Scotland, and all participants gave written informed consent.

In the SHeS Study, the participants were adults who took part in the Scottish Health Surveys 1995 and 1998. The surveys were based on a randomly selected, nationally representative, general population sample (see The Scottish Health Surveys websites for more detail) (117, 118). All participants were interviewed and asked questions about their health and lifestyle behaviours, and consent for measurement of weight and height and collection of blood sample was requested. The measurements of ferritin and outcome variables of WC, blood pressure, and HDL-C were available for 10,271 participants (59.4%). Glucose, triglycerides, HbA1C, and insulin were not measured. Among those with available measures of exposure and outcome variables, 8,879 had no missing values for covariates, which are listed in the data analysis section. I excluded children (individuals younger than 18 years old, n = 115), and 1998 data for individuals who took part in both SHeS surveys (n = 23). The final sample consisted of 8,653 people. The secondary analyses of the SHeS datasets were approved by the Ethics Research Subgroup from the Centre for Population Health Sciences of the University of Edinburgh.

Biochemical and clinical variables

Blood was collected via venepuncture after overnight fasting in the VIKING study, whereas in the SHeS, fasting samples were not collected, given that the only biochemical cardiovascular risk factors to be measured were cholesterol markers. After food intake, these markers of lipid profile appear to be, at most, minimally changed and have shown good prediction of increased risk for cardiovascular disease (119). In the VIKING study, glucose, triglycerides, and HDL-C were measured by using enzymatic-colorimetric methods. The same method was used for HDL-C in both the VIKING study and SHeS. Fibrinogen level was calculated from INR and APTT on a Sysmex CA560 in the VIKING study and by nephelometric method (clot turbidity) in the SHeS. Coulter analysers were used to measure haemoglobin, and the nitroanilide method was used for GGT in the SHeS. In the VIKING study, transaminases (GGT, aspartate transaminase [AST], and alanine transaminase [ALT]), insulin, haemoglobin, and HbA1C were measured by using standard Beckman-Coulter reagents in a Beckman-Coulter DxC600i combined chemistry and immunochemistry analyser.

In both the SHeS, blood pressure was measured using mercury sphygmomanometers with an appropriately sized cuff in a sitting position after 15 minutes of rest. Phase I and V (disappearance) Korotkoff sounds were used to identify SBP and DBP (120). In the VIKING study, digital sphygmomanometers were used. In the SHeS, three blood pressure readings were taken and the average of the second and third readings was used for the analyses. In the VIKING study, two readings after at least 10 minutes of supine rest were taken to calculate the average blood pressure. Body weight and height were measured using standard techniques and instruments (121). BMI was calculated as weight in kg/height in metres squared (122). WC was measured from the midpoint between the lateral iliac crest and the lowest rib using a flexible steel tape measure (123). The criterion of smoking included the following categories: never smoker, ex-regular or ex-occasional smoker, and current smoker. In the SHeS, the criterion of alcohol intake included the following categories, using rating units per week: 1) non-drinker or ex-drinker, 2) trivial drinker < 1 rating unit per week, and 3) drinker ≥ 1 rating unit per week. In the VIKING study, alcohol

intake had three categories: non-drinker or rarely, trivial drinker (special occasions or one to three days per month), and drinker (one or more days per week). Anaemia was defined as haemoglobin <13 g/dL in men and <12 g/dL in women (124). Overweight and obesity were defined as having a BMI ≥25 and BMI ≥30, respectively.

Metabolic syndrome, its components, and insulin resistance

Cut-off points from the international consensus definition for MetS were used as follows (40): high triglycerides (HTG) at ≥1.7 mmol/L, low HDL-C at <1.0 mmol/L in men and <1.3 in women, high fasting glucose (HFG) at ≥5.6 mmol/L, high blood pressure (HBP) as SBP ≥130 mm/Hg and/or DBP ≥85 mm/Hg (or antihypertensive drug treatment), and high WC ≥94 cm in men and ≥80 cm in women. MetS was defined as the presence of three or more variables meeting the definitions above. In the case of high glucose and triglyceride levels, these MetS components did not include additional cases with specific lowering treatment since this information was not available in the VIKING study. IR was estimated by homoeostatic model assessment using the formula: fasting glucose levels [mmol/L] × fasting insulin levels/22.5 (125), which was only available for the VIKING participants.

Data analysis

For both studies, medians and their interquartile ranges or proportions were used for description of continuous and categorical study variables, respectively, in the whole sample and by groups according to sex and menopausal status, and the differences were estimated via Mann-Whitney *U* test for continuous variables and by χ^2 test for categorical variables. Since both ferritin levels and cardiometabolic risk factors vary with sex and menopausal status, all the analyses of association between ferritin and MetS and its components were conducted by using logistic regression modelling in separate groups of men and pre- and post-menopausal women. Ferritin levels were used as a categorical variable of sex- and menopausal-specific quartiles and also as a continuous Z score of log-transformed ferritin. Associations between ferritin and MetS or its components were described before and after adjusting for potential confounders which were age, fibrinogen, GGT levels, smoking, alcohol consumption, and BMI. In the VIKING study, there was additional adjustment for

AST and ALT. For the SHeS, the year of the survey was also part of the adjustment since despite using consistent methods, there were changes in the laboratory where total and HDL cholesterol levels were measured in 1998. The set of confounding factors was chosen on the basis of possible influence of acute phase or subclinical inflammation, adiposity, and liver dysfunction on levels of ferritin and/or outcome variables. In the SHeS analysis, survey weights were used to adjust for disproportionate sampling, differing selection probabilities, and differential non-response. This weighting was applied in order to provide comparability with the studies on ferritin and MetS that used national survey data, all of which applied survey weights to produce nationally representative estimates of prevalence. More details of the survey weights in SHeS are provided in the data analysis section of chapter 5. In the VIKING study, the relationships between ferritin, IR, and HbA1C were additionally evaluated using multiple linear regression analyses to adjust for the potential confounders as listed above. Values of ferritin, HbA1C, HOMA-IR, fibrinogen, and transaminase were log-transformed to approximate to normal distribution before conducting linear regression analyses. I conducted sensitivity analyses on the basis of exclusion of individuals with anaemia, very high ferritin (>300 μg/L), potential inflammation/infection (fibrinogen > 4.7 g/L), and/or liver disease (ALT > 39 U/L, AST > 52 U/L, or GGT > 64 U/L). All analyses were processed using STATA 8.0 software (Statistics/Data Analysis, Stata Corporation, 4905 Lakeway Drive, College Station, TX 77845, USA, 800-STATA-PC).

3.2.2 Study 2: Systematic review and meta-analysis

Search strategy

Along with a fellow PhD student (Eduardo Ensaldo-Carrasco) I searched and selected articles from PubMed and EMBASE databases up to 31st August 2015. The following search terms were used: metabolic syndrome.mp. or metabolic syndrome X; ferritin or ferritin blood level or iron or body iron stores.mp. No restrictions regarding study design or article type were applied in the search, but unpublished reports were not considered. There were no disagreements about which studies to include between my colleague and me, so advice from a third researcher was not needed. Full texts and abstracts in English language regardless of language used in

the full text article were considered. The reference lists of the selected studies were read in order to find additional relevant studies. Although I intended to register the protocol of this systematic review/meta-analysis in the International Prospective Register of Systematic reviews "PROSPERO", this was not possible because PROSPERO requires protocols of systematic reviews to be registered before the extraction of data has started. The findings of this systematic review/meta-analysis are presented according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidance. The data extraction, risk assessment of bias and data analysis, which are described further below, were conducted by me alone.

Prevalence of MetS components were a secondary outcome in this systematic review/meta-analysis. However, I did not include specific search terms of MetS components (e.g. glucose, glycaemia, blood sugar levels, blood pressure) since in a systematic review conducted for my first year PhD review in 2014, studies on iron markers and individual MetS components or MetS-related variables that did not include MetS as outcome were heterogeneous in terms of effect estimates and adjustments (many of them unadjusted), which would have made a quantitative analysis difficult. Studies on MetS as outcome were used to describe associations with MetS components as well, so the individual association between ferritin and each MetS component was evaluated in those studies providing this additional information.

Study selection

Eligibility criteria were studies that included participants from the general adult population with descriptions of associations, stratified by gender and age groups or adjusting for these covariates at a minimum. Study populations exclusively composed of pregnant women, obese individuals, or people with a specific diagnosis were not considered. Studies of animals or genetic polymorphisms and reports of *in vitro* experiments were also excluded. The following selection issues were addressed by me alone. If two or more studies were based on the same population and same study design, I included the study with larger sample size. If the sample sizes were similar between studies of similar populations, I selected the study with more robust

adjustment. If there were two studies with the same population but with different designs, I selected both studies, but they were analysed separately (see more detail below in data analysis).

Data extraction and risk of bias

The data extracted from the selected articles were name of the study, publication year, country, time of survey or baseline survey, age (range or estimates), study design, sample size, percentage of male individuals, duration of follow-up, prevalence/incidence of MetS, MetS definition, ferritin levels, cut-off values for high ferritin, and covariates for adjustments. Additional data extracted consisted of exclusion criteria such as pregnancy/breastfeeding, anaemia, hepatic diseases, very high iron status, and inflammation/infection. Risk of bias was evaluated according to modified criteria of the Newcastle-Ottawa scale modified by Orban and Huth (19) in terms of representativeness, adjustment for confounders, description of exposure and outcome, and duration of follow-up (if prospective design) (Appendix Newcastle-Ottawa scale). The maximum and minimum scores were 7 and 0, respectively, and the higher the score the lower the likelihood of bias. Although representativeness is very complex to evaluate because a study can be representative of a specific location or group of people, I defined a study as representative if it was based on a national/regional health/nutrition survey, an epidemiological population-based study, or if, for instance, random selection was reported in the recruitment of participants.

Data synthesis and analysis

Odds ratios (ORs), hazard ratios (HRs) or relative risks (RRs), and their confidence intervals were extracted from the results described in the studies. Four studies describing mean ferritin levels reported by categories of MetS (yes/no) were retained for the systematic review but excluded from the meta-analyses. For these studies I did not use any method to derive the ORs for the association between mean ferritin levels and MetS, since these methods assume normal distribution of the variable, and distribution of ferritin is frequently skewed across diverse general populations (26, 126, 127). In addition, a meta-analysis of mean differences for the four studies was

not feasible owing to different effect estimates reported, in terms of normal mean, standardised mean, and mean of change in ferritin levels.

I decided to conduct the meta-analysis on ferritin and MetS by using two approaches: meta-analysis of cross-sectional studies and prospective studies [meta-analysis 1] and meta-analysis of cross-sectional studies only [meta-analysis 2]. The main rationale behind that decision was that there were few prospective studies, and it was necessary to ensure higher statistical power for sub-group and meta-regression analyses. The other reason was that few populations reported both cross-sectional and longitudinal associations, so it was relevant to determine the effect of both kinds of estimates on the pooled estimates and subgroup analyses. The meta-analysis on ferritin and MetS components did not require a similar approach since all of the studies describing associations between ferritin and MetS components were cross-sectional in design, with the only exception being Vari et al. who reported both cross-sectional and prospective associations(7). I used cross-sectional findings from the study by Vari et al. for this meta-analysis of the association between ferritin and MetS components.

I pooled estimates from the studies by using an inverse-variance weighted random-effects model. The I^2 statistic was used to estimate heterogeneity in terms of the proportion of total variation in the estimates of meta-analysis explained by heterogeneity. For the meta-analysis 1 of cross-sectional and prospective studies, because most of the studies provided OR as effect estimate, hazard ratios, ORs and relative risks were assumed to approximate the same effect estimate of OR. I conducted sub-group and meta-regression analyses to evaluate the potential factors accounting for heterogeneity in the associations between ferritin-MetS and between ferritin-Mets components throughout the selected studies. The factors were: study design (cross-sectional or prospective), type of effect estimate (OR, HR, relative risk), geographic region (Asia, Europe, America), adjustment for BMI (yes/no), adjustment for CRP (yes/no), adjustment for any inflammatory marker (yes/no), adjustment for hepatic function markers (yes/no), sample size (<500 or ≥500), sample size (<1000 or ≥1000), ferritin assay (quimiolumominescence QLA,

radiometry, RIA; inmunoturbidimetry, TIA; others), average ferritin levels reported, and cut-off points reported for the highest category of ferritin levels. For these latter two factors, I calculated tertiles and quartiles specific of sex and menopausal status or whole population as reported in each study selected. The VIKING and SHeS studies were assigned to the corresponding tertile and quartile groups according to how their values fitted in the ranges of those categories. I did not use data extracted on exclusion criteria of the studies for additional subgroup analyses, to avoid higher likelihood of findings by chance. Effect estimates of the VIKING study were part of the meta-analyses for MetS and its components, and findings from the SHeS were included in the meta-analysis for MetS components only. Publication bias was evaluated by using Begg's and Egger's test as well and visualisation of funnel plots. All analyses were processed using STATA 14.0 software (Statistics/Data Analysis, Stata Corporation, 4905 Lakeway Drive, College Station, TX 77845, USA, 800-STATA-PC).

3.3 Results

3.3.1 Study 1: Viking Health study – Shetland and SHeS 1995-1998

Differences between selected and excluded individuals from the VIKING study and the SHeS 1995–1998 are shown in Appendices Tables 10 and 11, respectively. Individuals included in the analysis of the VIKING study had slightly higher values of ferritin and GGT and lower values of HDL than the subjects who were excluded because of missing data. In the SHeS 1995–1998, most of the study variables were significantly different between included and excluded subjects but without a clear pattern or trend for higher or lower values of risk factors and covariates in one group or the other. Compared to the people that were excluded the people that were included were slightly older; had smaller proportions of postmenopausal women and higher proportions of men; and higher values of BMI, HDL-C, and DBP. On the other hand, the people who were excluded had higher values of GGT and fibrinogen and higher proportions of current smokers and people with CVD and diabetes than those who were included.

Table 7 describes the study variables from the VIKING study and SHeS. The population in the VIKING study was older (median (IQR) age in years [51(38.9–63.2) vs. 40(31–52)]) and had higher values of DBP and WC than the SHeS population. The SHeS participants had higher values of GGT and a higher proportion of current smokers than VIKING participants (Table 7). Prevalence of overweight was 56.2% and 66.8% in VIKING and SHeS, respectively. Obesity was present in 18.2% of the individuals from SHeS and in 24.4% from the VIKING study. The distribution of other common variables between the studies appeared to be similar.

Ferritin levels (median and interquartile range) were statistically significantly higher in subjects with MetS in each sex and menopausal status category [Premenopausal women: MetS (prevalence 8.6%) 31(16-64) µg/L v. No MetS 25(15-40) µg/L, P= 0.047; Postmenopausal women MetS (prevalence 22 %) 68(32-95) µg/L v. No MetS 57(37-78) µg/L, P=0.016; Men MetS (prevalence 22.2%) 111(63-163) µg/L v. No MetS 87(57-129) µg/L, P= 0.001].

Table 7 Distribution of iron status and cardiometabolic risk in the study cohorts

	Viking Health study – Shetland (2013-2015)	Scottish Health Surveys (1995 & 1998)
n	2047	8653
Age (years)	51(38.9-63.2)	41(31-52)
Pre-MW/ Post-MW/ Men %	28.7/30.5/40.6	33.6/20.1/46.3
BMI (kg/m^2)	26.7(24.1-29.9)	25.6(23.1-28.6)
Ferritin(µg/L)	56(29-97)	62(33-108)
Fibrinogen (g/L)	3.3(2.9-3.8)	2.9(2.4-3.5)
GGT U/L	16(12-24)	20(14-32)
ALT U/L	22(17-29)	N.M
AST U/L	22(19-26)	N.M
HDL-C (mmol/L)	1.4(1.2-1.7)	1.4(1.2-1.7)
SBP (mmHg)	127(116-142)	126(117-137)
DBP (mmHg)	75(69-82)	70(63-79)
WC (cm)	91.2(81.5-100.8)	85.2(76.3-94.2)
Glucose (mmol/L)	4.8(4.5-5.1)	N.M
TG (mmol/L)	0.9(0.6-1.3)	N.M
Insulin mU/mL	37.1(25.8-54)	N.M
HOMA-IR	7.8(5.3-11.8)	N.M
HbA1C (%)	5.30(5.10-5.50)	N.M
MetS and its components n (%)*		
High blood pressure	1032(50.4)	3783(41.9)
Low HDL-C	350(17.1)	1646(18.4)
High WC	1366(66.7)	3651(40.0)
High glucose	147(7.2)	
High triglycerides	244(11.9)	
MetS	374(18.3)	
Smoking n(%)		
Never smoker	1133(55.3)	3349(39.9)
ex-regular or ex-occasional smoker	747(36.5)	2376(27.2)
Current smoker	167(8.2)	2928(32.9)
Alcohol consumption n(%)		
Category 1*	84(4.1)	687(7.1)
Category 2*	152(7.4)	1001(10.1)
Category 3*	1811(88.5)	6965(82.7)
Cardiovascular disease n(%)	64(3.1)	206(2.0)
Diabetes n(%)	46(2.2)	153(1.5)

Data are median (interquartile range) or n(%). Comparison between groups by Mann-Whitney U and χ^2 test. N.M, not measured. Pre-MW, premenopausal women. Post-MW, postmenopausal women. BMI, body mass index. TG, triglycerides. SBP, systolic blood pressure. DBP, diastolic blood pressure. WC, waist circumference. HOMA-IR, homeostatic model assessment insulin resistance. * VIKING: non-drinker or rarely, trivial drinker (special occasions or 1 to 3 days per month), and drinker (1 or more days per week) ; SHeS 95-98: 1) non-drinker or ex-drinker, 2) trivial drinker < 1 rating unit per week and 3) drinker ≥ 1 rating unit per week. * For the SHeS study *n* is on the basis of unweighted values but percentage(%) is weighted by survey

weights to produce nationally representative estimates of prevalence. More details of the survey weights in SHeS are provided in the data analysis section of chapter 5.

In unadjusted analyses, high ferritin levels were associated with MetS in men and postmenopausal women of the VIKING study (Table 8). In terms of MetS components, there were also several significant positive crude associations of ferritin in the highest quartile compared to the lowest quartile with MetS components in the VIKING and SHeS studies, mainly in men and postmenopausal women (Appendix table 12). However, the above associations between ferritin, MetS, and some MetS components did not remain significant after adjusting for covariates, with the exception of the associations: ferritin-HDL-C in postmenopausal women, ferritin-WC in men (SHeS) and postmenopausal women (VIKING) (Table 8, Appendix Table 13). On the other hand, in premenopausal women from the SHeS, ferritin levels in the middle quartiles (2 and 3) were significantly and inversely associated with high blood pressure in unadjusted and adjusted analyses. Given this observation, I furtherly examined a potential U or J-shape association using quartiles 2 and 3 together as reference. Both quartiles 1(Q1) and 4(Q4) of serum ferritin were positively associated with high blood pressure in premenopausal women although only the association with quartile 1 reached statistical significance, $P< 0.05$ [ferritin Q1 v. Q2-3 fully adjusted OR(95%CI) 1.49(1.14-1.94) P=0.003; ferritin Q4 v. Q2-3 fully adjusted OR(95%CI) 1.23(0.94-1.61) P=0.116].

A similar pattern of no association with MetS was observed when using serum ferritin as a continuous variable (Z score of log-ferritin) (Table 9). There were no additional significant associations between the ferritin Z score and each MetS component to those reported by using ferritin quartiles ($P > 0.05$), with the exception of a significant association with increased WC in postmenopausal women in SHeS [unadjusted OR (95% CI) 1.56 (1.29–1.88) $P < 0.001$, adjusted OR (95% CI) 1.53 (1.07–1.51) $P = 0.005$] (Appendix table 14).

In the VIKING study, ferritin levels were inversely associated with HbA1C in unadjusted and adjusted linear regression models in men and both groups of women

of the study (Appendix Table 15). HOMA-IR, although significantly associated in unadjusted models, was not independently associated with ferritin levels, even after increasing statistical power by considering the whole sample of the VIKING study with adjustment for sex and menopausal status (Appendix Table 15).

I conducted additional adjustments for prevalent cardiovascular disease and diabetes but this did not substantially modify the effect estimates for the associations previously described in the VIKING and SHeS studies.

In the VIKING study, the exclusion of cases of iron deficiency (n= 183, 8.9%), or serum ferritin >300μg/L (n=38, 1.8%) did not affect the above associations. The same was true when individuals with potential inflammation (high fibrinogen levels) (n= 160, 7.8 %) and/or liver disease (n=147, 7.1%) were removed from the analyses, with the exception of two associations. One of them was a significant association between ferritin Z score and low HDL-C in men, not observed in the previous findings [unadjusted OR (95% CI) 1.57 (1.15–2.13) P = 0.004, adjusted OR (95% CI) 1.39 (1.0009–1.92) P = 0.044]. The other one was the relationship between ferritin and HOMA-IR in the VIKING study, which became significant after adjustments but only in men [Beta (95% CI) -0.07 (-0.13 to -0.01) P = 0.019]. However, the above associations presented confidence intervals containing values of adjusted O.R and Beta coefficient too close to 1.00 and zero, respectively, which implies that these associations may not exist or are too small to be of clinical relevance. In addition, the adjusted ferritin-HOMA-IR association was negative in contrast with a positive effect estimate in the unadjusted regression [Beta (95% CI) 0.03 (-0.03 to 0.10) P = 0.382]. This may be a chance finding.

In the SHeS study, the few significant associations between ferritin and some MetS components reported previously in Appendix Table 13 were attenuated in sensitivity analyses. Lower statistical power might have been the cause, since both effect estimates and statistical significances decreased. In the SHeS, when individuals with high ferritin (n= 631, 7.2%) were removed, the significant association between high serum ferritin and high WC in men was attenuated [adjusted OR (95%CI) 1.25(0.92-

1.70), P=0.138]. Removal of subjects with potential inflammation (n=680, 7.8%) and/or liver disease (n=270, 3.1%) weakened the same association in men [adjusted OR (95%CI) 1.22(0.89-1.68), P= 0.207], and also the significant inverse ferritin-HDL-C association reported in postmenopausal women [adjusted OR (95%CI) 1.41(0.87-2.28), P= 0.158]. Exclusion of subjects with iron deficiency (n= 631, 7.2%) did not alter any of the findings described for the whole sample. No other substantial modifications were observed in sensitivity analyses.

Adjustment for BMI had a major effect on the association between high ferritin and MetS in the VIKING study and on the associations between high ferritin and increased WC, blood pressure, and low HDL-C in the SHeS. Adjustment for age and GGT levels also attenuated the associations with increased WC and blood pressure in the SHeS.

Table 8 Odds ratios (95% CI) for MetS by sex- and menopausal-specific quartiles (Q) of serum ferritin in the VIKING study

	Unadjusted					**Adjusted***			
	Q1	Q2	Q3	Q4	P for trend	Q2	Q3	Q4	P for trend
Premenopausal women	≤ 15 µg/L	16-25 µg/L	26-41 µg/L	> 41 µg/L		16-25 µg/L	26-41 µg/L	> 41-67.1 µg/L	
	1.0 (Ref)	0.99 (0.43-2.28)	0.39 (0.13-1.14)	1.99 (0.94-4.20)	0.129	1.00 (0.39-2.54)	0.27 (0.08-0.92)	1.02 (0.42-2.46)	0.772
Postmenopausal women	≤ 34 µg/L	35-54 µg/L	55-84 µg/L	> 84 µg/L		35-54 µg/L	55-82 µg/L	> 84-450 µg/L	
	1.0 (Ref)	0.37 (0.20-0.71)	0.88 (0.52-1.50)	1.46 (0.89-2.39)	**0.024**	0.43 (0.22-0.87)	0.57 (0.31-1.03)	1.09 (0.62-1.90)	0.606
Men	≤ 58 µg/L	59-89 µg/L	90-138 µg/L	>138 µg/L		35-55 µg/L	90-138 µg/L	>138 -772 µg/L	
	1.0 (Ref)	0.96 (0.58-1.58)	1.24 (0.76-2.03)	**2.15 (1.36-3.41)**	**<0.001**	1.16 (0.65-2.06)	1.17 (0.66-2.06)	1.43 (0.83-2.46)	0.207

*** Adjusted for age, alanine-aminotransferase levels, aspartate-aminotransferase levels, glutamyl-transferase levels, fibrinogen levels, smoking, alcohol consumption, and BMI. Ref, reference. Significant associations are shown in bold (P<0.05). Q, quartile.**

Table 9 Unadjusted and adjusted odds ratios (95% CI) for metabolic syndrome by SD units of serum ferritin (log-transformed) in the VIKING study

	Unadjusted		**Adjusted***	
	Ferritin Z score	P value	Ferritin Z score	P value
Premenopausal women	**1.44(1.06-1.96)**	**0.018**	1.05(0.75-1.44)	0.789
Postmenopausal women	**1.26(1.03-1.53)**	**0.022**	1.07(0.86-1.33)	0.491
Men	**1.27(1.07-1.51)**	**0.005**	1.07(0.89-1.28)	0.464

*** Adjusted for age, alanine-aminotransferase levels, aspartate-aminotransferase levels, glutamyl-transferase levels, fibrinogen levels, smoking, alcohol consumption, and BMI. Significant associations are shown in bold (p<0.05).**

3.3.2 Study 2: Systematic review and meta-analysis

Studies with the same population and decisions made

Lee et al. (108), Cho et al. (128), Kang et al. (129), and Yoo et al. (130) conducted studies using the Korean National Health and Nutrition Examination Survey (KNHANES) but at different times. The latter two studies used mixed populations from the 2007 and 2008 surveys, whereas Lee et al. described the 2007 data, and Cho et al. described the 2008 data. The study by Yoo et al. was excluded because it has a smaller sample size than the one by Kang et al. I confirmed with the Korea Centres for Disease Control and Prevention, that the possibilities for samples from 2007 and 2008 surveys to overlap were minimal since only about 5 households have been surveyed twice between 2007 and 2015 in the KNHANES. Therefore, I decided to include the studies by Lee et al. and Cho et al. instead of the one by Kang et al., which had a slightly larger sample size but included overlapping populations with the above studies. This strategy allowed me to include two studies from independent Korean populations in the meta-analysis, providing more cases than one single study. Additionally, the study by Cho et al., specifically involving women, provided some balance against the large proportion of articles selected involving male populations only.

Han et al. (131) and Li et al. (132) used data from the China Health and Nutrition Survey, 2009, and had comparable samples sizes (1.4% difference in sample size between study populations). I selected the study by Li et al. for its robust statistical adjustment.

The cross-sectional study by Ryoo et al. (133) and the longitudinal study by Park et al. (134) included some similar subjects/cases, so they were entered separately into the meta-analyses.

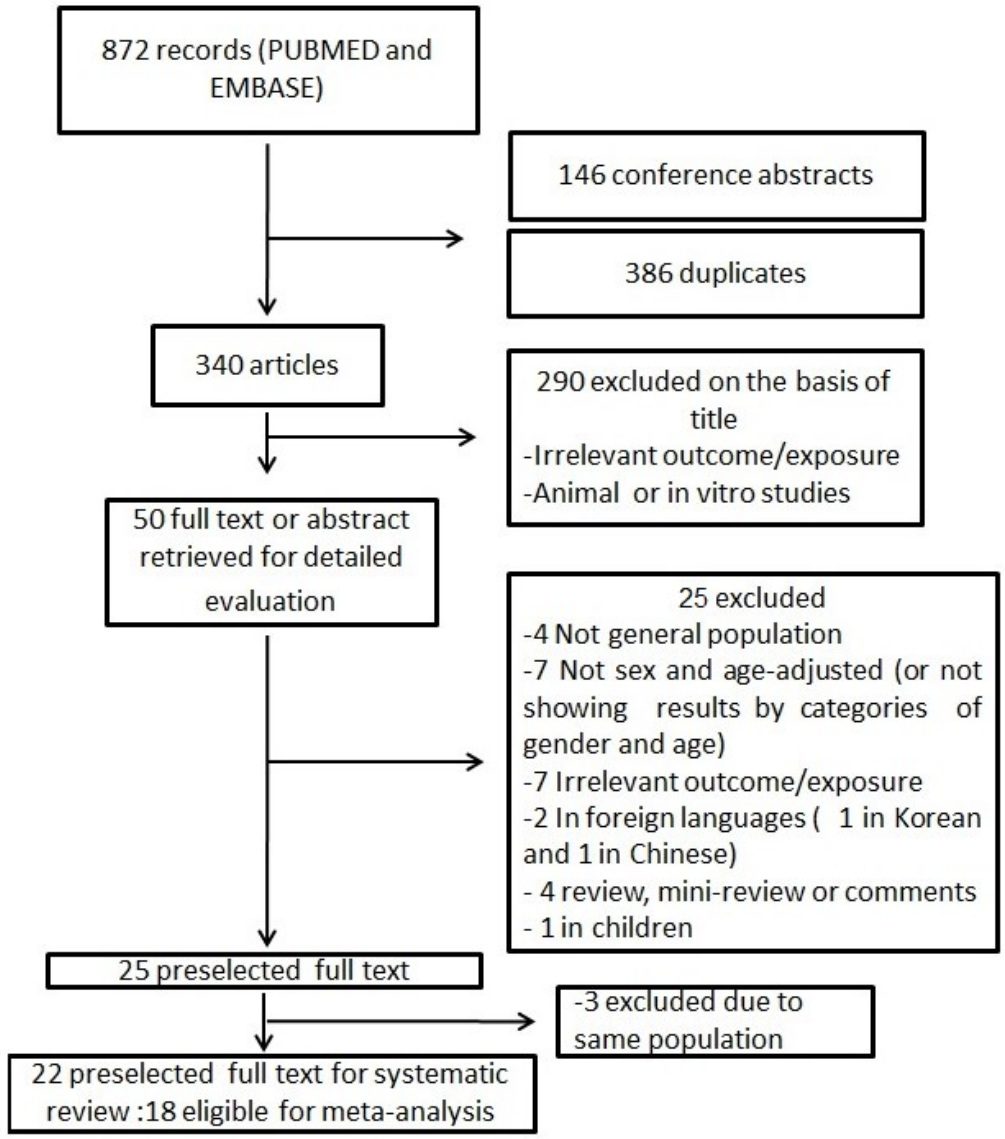
Hamalainen et al. and Kilani et al. published both cross-sectional and longitudinal population-based studies from Pieksamaki (Finland) (113, 135) and from Lausanne (Switzerland) (22, 136), and both sets of analyses were included for the appropriate parts of the present study.

There were only two studies in languages other than English, i.e. Chinese and Korean cross-sectional studies by Xiao et al. (137) and Ryu et al. (138), respectively. Coincidently, another study by Yoon et al. was selected, as it was conducted in the same cohort of the study by Ryu et al. (Korean Rural Genomic Cohort) but with prospective design and written in English language.

Studies selected

Figure 4 summarises the process of identifying and selecting the studies. We identified 22 studies that described the association between ferritin and MetS of which 18 were included in the meta-analyses and systematic review and four only contributed to the systematic review (Table 10). The VIKING study provided a further study to include in the ferritin-MetS meta-analysis. Among the studies selected, nine also provided information on associations between ferritin and MetS components, and thus along with the VIKING study and SHeS, there were 11 studies for the meta-analysis on the association between ferritin and MetS components.

Figure 4 Flow chart for the selection of eligible studies included in the systematic review/meta-analysis of the association between ferritin and metabolic syndrome



MetS definitions, geographic location, and types of source

Among the studies selected (Table 10), with regards to MetS definitions, 14 exclusively used NCEP ATP III criteria, of which seven had modifications of the WC criterion or used revised criteria for Asian or Asian-American individuals. One study specifically used the IDF criteria, another used the Japanese criteria, six used the harmonised definition (including the VIKING study), and one used three definitions (NCEP ATP III, IDF, and revised NCEP ATP III criteria). Three studies were prospective only, and two reported cross-sectional and prospective evaluations. The rest of the studies were cross-sectional analyses. Thirteen studies were

conducted in Asian populations, eight in Europe, one in Israel, and one in the US. Nine used information from national/regional surveys of health or nutrition, five involved samples from population-based studies, three involved participants identified from medical health centres or social security numbers, two involved employees who underwent an annual health check-up, two involved a cohort of subjects born in specific years, and two involved individuals from a factory and six offices. The SHeS study, which was not used for the meta-analysis of MetS but for that of MetS components, used criteria from the harmonised MetS definition, reported cross-sectional associations, and used information from a national survey (Table 10).

Table 10 Characteristics of the studies included in the systematic review and meta-analysis and in the systematic review only of the association between ferritin and metabolic syndrome (seven pages table)

STUDIES INCLUDED IN THE SYSTEMATIC REVIEW AND META-ANALYSIS											
Authors, year (Ref)	**Study**	**Location/ Universe**	**Study-Survey /Year of survey**	**Age range (years)***	**Male (%)**	***n***	**Prevalence of metabolic syndrome**	**MetS definition**	**Adjustments**		
									BMI	**CRP**	**Other adjustments**
Jehn et al., 2004(106)	Cross-sectional	U.S/ U.S population	NHANES III /1988-1994	≥20	20.1	6044	17.5% (Men), 10.2% (Premenopausal women), and 27.8% (postmenopausal women)	NCEP ATP-III	yes	yes	Alcohol intake and smoking
Vari et al.,2007(7)	Cross-sectional/Prospective(6 years follow-up)	France/Users insured by French Social Security	DESIR/NP	30-65	49.7	944	21% (Men), 8% (Premenopausal women), and 24% (postmenopausal women)	IDF NCEP ATP-III NCEP revised	No	No	None
Zelber-Sagi et al., 2007(139)	Cross-sectional	Israel	First Israeli National Health and Nutrition Survey/2003-2004	24-70	52.7	349	NP as a total	NCEP ATP-III	No	No	Non-alcoholic fatty liver disease
Shi et al., 2008(107)	Cross-sectional	China/China population	National Nutrition Survey /2002	>20	45.9	1294	9.4%(men) and 18%(women)	IDF	No	No	Residence (urban/rural), education level, and income
Sun et al., 2008(140)	Cross-sectional	China/ China	NHAPC/ 2005	50-70	43	3289	42.3%	NCEP ATP-III	Yes	Yes	Alcohol intake, smoking, family history of chronic

STUDIES INCLUDED IN THE SYSTEMATIC REVIEW AND META-ANALYSIS											
Authors, year (Ref)	**Study**	**Location/ Universe**	**Study-Survey /Year of survey**	**Age range (years)***	**Male (%)**	***n***	**Prevalence of metabolic syndrome**	**MetS definition**	**Adjustments**		
									BMI	**CRP**	**Other adjustments**
											diseases, dietary factors, IL-6, TNF-R2, adiponectin, PAI-1, and RBP4)
Cho et al., 2011(128)	Cross-sectional	Korea/ Korea population	KNHANES /2007	36.9 ± 8.2 (Premenopausal) and 64.8 ± 9.5 (Postmenopausal)	0 (1691 and 1391 pre and postmenopausal women, respectively)	3082	10.6 (Premenopausal) and 41.9 (Postmenopausal)	NCEP ATP-III and the Korean Society for Study of Obesity (WC cut-off points)	Yes	No	HOMA-IR, alcohol intake, smoking history, exercise, intake of energy, iron, hemoglobin, ASAT, ALAT, and hormone therapy use (postmenopausal women)
Kim et al., 2011(141)	Cross-sectional	Korea/ Users of a Health Promotion Centre in Seoul	NP/2008	20-89	52.7	12090	NP	NCEP ATP-III	Yes	Yes	Smoking, alcohol use, and menopause status (women).
Lee et al.,2011(108)	Cross-sectional	Korea/ Korea population	KNHANES IV /2008	>20	42.5	6311	16.3%(Men), 9.5% (Premenopausal women), and 31.5%(postmenopausal women)	NCEP ATP-III and the Korean Society for Study of Obesity (WC cut-off points)	Yes	No	Alcohol intake, smoking, educational level, AST and ALT.
Ryoo et	Cross-	Korea/	NP/2008	40.5 ±	100	1858	13.8	NCEP ATP-III	No	No	Alcohol intake,

STUDIES INCLUDED IN THE SYSTEMATIC REVIEW AND META-ANALYSIS											
Authors, year (Ref)	**Study**	**Location/ Universe**	**Study-Survey /Year of survey**	**Age range (years)***	**Male (%)**	***n***	**Prevalence of metabolic syndrome**	**MetS definition**	**Adjustments**		
									BMI	**CRP**	**Other adjustments**
al., 2011(133) **	sectional	Employees from companies in an Annual health check-up		6.5		1					recent smoking status, total protein, GGT, log(hsCRP), WBC, ALT, ApoB, TIBC, serum creatinine and HOMA-IR
Yoon et al., 2012(105)	Prospective (5 years follow-up)	Korea/Korean Rural Population	Korean Genomic Rural Cohort/NP	>40	49.8	861	13.3	Harmonized definition	No	Yes	HOMA-IR, adiponectin, leptin, ALT, exercise, alcohol intake and smoking status
Park et al., 2012(134)	Prospective (5 years follow-up)	Korea/ Employees from companies in an Annual health checkup	NP/2005-2010	30-59	100	1902 2	16.3	Harmonized definition	No*	Yes	WBC, GGT, HOMA-IR, serum creatinine, TIBC, smoking status, regular exercise, alcohol intake, hypertension, diabetes
Chang et al.,2013(142)	Cross-sectional	Taiwan/ Taiwan population	NAHSIT /2005-2008	≥19	47.4	2654	43.1%(Men), 26.5% (women)	NCEP ATP-III for Asia Pacific	Yes	No	GOT, GTP, ALK, Amylase, BUN, UA, creatinine, homocysteine, past smoker, alcohol intake, betel nut intake,

STUDIES INCLUDED IN THE SYSTEMATIC REVIEW AND META-ANALYSIS											
Authors, year (Ref)	**Study**	**Location/ Universe**	**Study-Survey /Year of survey**	**Age range (years)***	**Male (%)**	***n***	**Prevalence of metabolic syndrome**	**MetS definition**	**Adjustments**		
									BMI	**CRP**	**Other adjustments**
											haemoglobin, iron deficiency anemia, and family history of chronic diseases
Li et al. , 2013(132)	Cross-sectional	China/China population	CHNS /2009	≥18	46.6	8441	19.9%(Men), 25.4% (women)	NCEP ATP-III for Asia Americans	No	Yes	Nationality, alcohol intake and smoking
Kilani et al., 2014(136)	Cross-sectional	Switzerland/ Population from Lausanne	The Cohorte Lausannoise/ 2003-2006	35-75	47.2	5498	29.4%(Men) 8.3%(premenopausal women) and 25.5%(postmenopausal women)	NCEP ATP-III	Yes	Yes	Alcohol intake, smoking, iron supplement and altered hepatic markers
Ledesma et al., 2015(143)	Cross-sectional	Spain/ workers from a factory in Zaragoza	The Aragon Workers' Health Study/2009-2019	19-65	100	3386	27.1	Harmonized definition	No	No	History of blood donations, alcohol intake and transaminases
Seo et al., 2015(144)	Cross-sectional	Korea/ Users of a health promotion center in Seoul	NP/2008-2010	>40	0	280	25~%	NCEP ATP-III (BMI used instead of waist circumference)	No	No	Alcohol intake, haemoglobin, transaminases and hormone status (E2, total testosterone, FSH, and TSH)
Tang et al., 2015(145)	Cross-sectional/Prospective(4 years	China/ Men from Guangxi	Fangchenggang Area Males Health and Examination	17-88	100	2417	Prevalence :12.7 % Incidence: 9.42%	NCEP ATP-III for Asia Americans	Yes	No	Physical activity, family history of chronic diseases, alcohol intake and

STUDIES INCLUDED IN THE SYSTEMATIC REVIEW AND META-ANALYSIS											
Authors, year (Ref)	**Study**	**Location/ Universe**	**Study-Survey /Year of survey**	**Age range (years)***	**Male (%)**	***n***	**Prevalence of metabolic syndrome**	**MetS definition**	**Adjustments**		
									BMI	**CRP**	**Other adjustments**
	follow-up)		Survey/2009-2013								smoking status
Kilani et al., 2015(22)	Prospective (5.5 years follow-up	Switzerland/ Population from Lausanne	The Cohorte Lausannoise/ 2003-2006	35-75	42.8	3271	22.6%(Men), and 16.5 % (women	NCEP ATP-III	Yes	Yes	Alcohol intake, smoking, iron supplement and altered hepatic markers
VIKING (This study)	Cross-sectional	Scotland	Viking Health Study-Shetland/2013-2015	18-92	40.7	2047	18.3%	Harmonized definition	Yes	Yes	Fibrinogen levels, GGT and AST levels, smoking and alcohol consumption
SHeS 95&98 (This study)*	Cross-sectional	Scotland	Scottish Health Surveys 1995 and 1998	18-74	47.9	7046	Not apply	*MetS was not evaluated but criteria for high blood pressure, increased waist circumference and low HDL-C from the Harmonized definition were used*	Yes	Yes	Fibrinogen levels, GGT levels, smoking and alcohol consumption

STUDIES INCLUDED ONLY IN THE SYSTEMATIC REVIEW											
Authors	**Study**	**Location/ Universe**	**Study-Survey /Year of survey**	**Age range (years)**	**Male (%)**	***n***	**Prevalence of metabolic syndrome**	**MetS definition**	**Adjustments**		
									BMI	**CRP**	**Others adjustments**
Martinelli et al.,2012(25)	Cross-sectional	Italy/ Val Borbera population	Val Borbera/NP	>18	44.3	1391	21.9%	Harmonized definition	No	Yes	C282Y HFE mutation, haemoglobin, uric acid, and creatinine
Hamalainen et al.,2012(135)	Cross-sectional	Finland/Middle –aged subjects from Pieksamaki who were born in 1942,1947,1952,1957 or 1962	NP/2003-2004	52.1 ± 6.2 years(men) and 52.1 ± 6.2 years (women)	44.5	766	53%(men), 40% (women)	NCEP ATP-III	No	Yes	Smoking, alcohol intake and physical activity
Hamalainen et al.,2014(113)	Prospective (6.5 years follow-up)	Finland/Middle –aged subjects from Pieksamaki who were born in 1942,1947,1952,1957 or 1962	NP/1998-2004	45.3 ± 6.2 years(men) and 45.1 ± 6.5 years (women)	41.8	691	Incidence :18%	Harmonized definition	Yes	Yes	Smoking, alcohol intake and physical activity

Iwanaga et al., 2011(146)	Cross-sectional	Japan/in individuals from a worksite lifestyle intervention study	NP/2007	41.2 ± 10.4 years	42.7	685	13.6 (men), 1.7 (women)	Japanese criteria	No	No	None

*** Or mean (SD) of age if age range not provided. ** This study used BMI instead of waist circumference as surrogate for central obesity. *** The SHeS findings were only used in meta-analysis on ferritin and individual components of MetS. Ref, reference. NP, Not provided.**

Adjustments

One study exclusively involved post-menopausal women (144), another, only women (both pre and post-menopausal) (128), and four, only men (133, 134, 143, 145). Information on adjustment variables used in the studies is shown in Table 10. Since basic adjustments for age and sex were the inclusion criteria for this systematic review, all of the studies showed either adjustments or stratified results for age and sex Twelve studies, including the VIKING study and SHeS, reported adjustment for BMI (22, 106, 108, 113, 128, 136, 140-142, 145), 11 for CRP levels as marker of sub-clinical/clinical inflammation, (22, 25, 105, 106, 108, 113, 132, 134-136, 140, 141), and 6 reported adjustments for both covariates (22, 106, 113, 136, 140, 141). One from those with no covariate of CRP, adjusted for white blood cell count or other inflammatory markers (133), and in the two Scottish studies, fibrinogen levels were used as inflammatory marker. Eleven studies and the two studies involving Scottish populations reported adjustments for hepatic function in terms of transaminase levels (22, 105, 108, 128, 134, 136, 141-144) or non-alcoholic fatty liver disease (139), two, for family history of chronic diseases (140, 142), and four, for HOMA-IR (105, 128, 133, 134), of which three did not adjust for BMI (105, 133, 134). With the exception of six studies (7, 25, 107, 135, 139, 146), all others adjusted for alcohol intake. Two articles included education level as covariate (107, 108), out of which one additionally adjusted for variables such as urban or rural residence and income (107). However, this latter study did not adjust for other factors.

Exclusion criteria

As complementary information for the adjustments mentioned above, Appendix Table 16 lists the exclusion criteria for each study. From 17 studies that included pre-menopausal women, only four described pregnancy as an exclusion criterion (106-108, 139).

Five studies excluded subjects with inflammation by clinical evaluation and high levels of CRP or WBC (7, 106, 140, 141, 143) (Appendix Table 16), and among these, three had further adjustment for an inflammatory marker in the analyses (106, 140, 141). Nine of the remaining 17 articles with no exclusion of subjects with

evidence of active inflammation, included adjustment for some inflammatory marker (22, 25, 105, 113, 132-136). Hepatic disorders based on infectious and/or chronic diseases and/or high levels of transaminases or transferases were the exclusion criteria in six studies (106, 108, 133, 134, 141, 145), of which three had further adjustment for a hepatic function marker. Half of the 16 studies with no exclusion of individuals with evidence of hepatic disorders adjusted for levels of transaminases or transferases (22, 105, 128, 136, 139, 142-144). Four studies excluded subjects with anaemia or iron deficiency (7, 106, 108, 141), adjustment for anaemia and/or haemoglobin levels was conducted in four studies (25, 128, 142, 144), two more adjusted for use of iron supplements (22, 136), and in another study, anaemia was an additional exposure variable (107) (Appendix Table 16). Subjects with probable HH were excluded in 11 studies on the basis of high levels of iron status markers (mostly high ferritin) (7, 22, 106, 108, 133, 134, 136, 140, 142, 143, 145), and one more study excluded confirmed homozygote individuals for C282Y mutation in the HFE gene and adjustment for heterozygote status for this mutation was conducted in the same study (25) (Appendix Table 16).

Only six studies excluded patients with cardiovascular disease and/or hypertension (105, 133, 134, 141, 144, 146) and among these, three additionally excluded subjects under diabetic and/or lipid/blood pressure lowering treatments (105, 134, 146) (Appendix Table 16). Of note, two of these three latter studies were prospective (105, 134) and their rationale for exclusion of those under medications was mainly that of excluding cases with MetS components such as high blood pressure, glucose, and triglycerides at baseline. However, one of these prospective studies further adjusted the associations for diabetes status (134). The rest of studies with no exclusion of people with the above diseases did not report stratified results or adjustment for diabetic and/or cardiovascular disease status. Four of the six prospective studies excluded individuals with MetS at baseline (22, 105, 134, 145) (Appendix Table 16) whereas the remaining two adjusted or stratified for having MetS at baseline (7, 113).

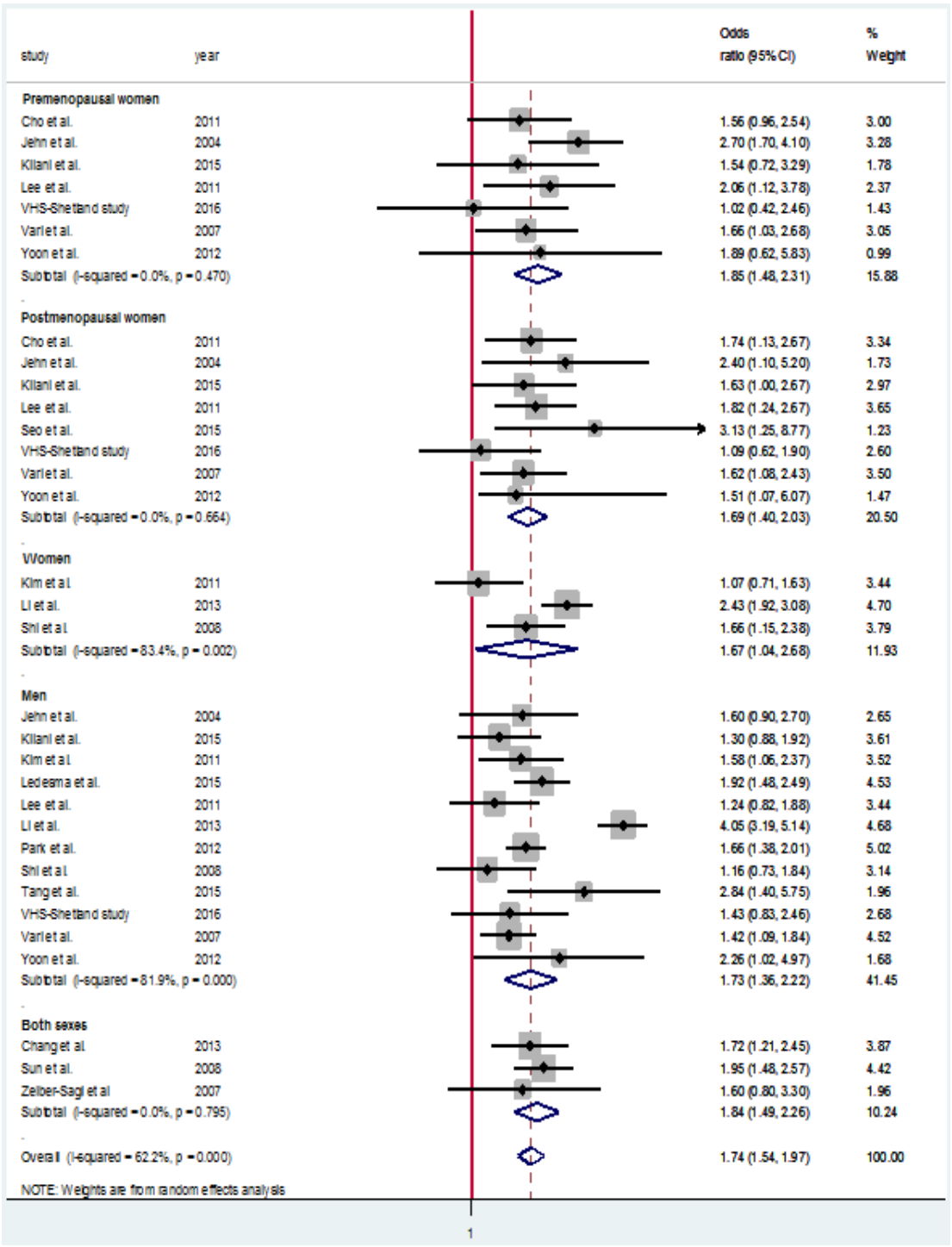
Average ferritin concentrations and cut-off values defining high ferritin

Median/mean values of ferritin levels and cut-offs of ferritin defining high concentration reported in the studies selected are shown in Appendix Tables 17 and 18 for meta-analyses 1 and 2, respectively. The values are grouped by sex/menopausal status/sex-specific tertiles and quartiles. The values for the Scottish studies were located in the lowest categories of average ferritin concentrations and cut-offs for high ferritin (Appendix Tables 17 and 18). All of the studies described cut-offs for high ferritin lower than suggested reference values (>200 µg/L in women, >300 µg/L in men) (18), with the exception of Kilani et al. (326 µg/L in men) (22, 136) and Tang et al. (459.9 [cross-sectional study] and 426.6 µg/L [prospective study] in men) (145).

Risk of bias

Appendix tables 19 and 20 describe our evaluation of risk of bias in cross-sectional and prospective studies, respectively. The median score for risk of bias, which is inversely related to opportunity of bias, was 4. Two cross-sectional studies, i.e. Sun et al. and Jehn et al., reached the maximum possible score of 7 for lower risk of bias (Appendix Table 19). Of note, many studies with very robust adjustments did not obtain high scores, presumably because one of the assessment criteria was the simultaneous adjustment for BMI and inflammatory markers. Failure to report coefficients of variation in ferritin measurements was another common reason for not obtaining higher scores (Appendix Tables 19 and 20).

Figure 5 Forest plot describing the association between ferritin and metabolic syndrome in observational studies (cross-sectional and prospective [Meta-analysis 1]).



Studies are stratified by sex, menopausal status or presented both sexes depending on the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models. VHS-Shetland study = VIKING study.

Ferritin and metabolic syndrome: Results of the meta-analysis and meta-regression

Information from 81,801 individuals was obtained when cross-sectional and prospective studies were analysed together (meta-analysis 1; 17 studies). The pooled OR for MetS by high levels of ferritin (vs. lowest levels) was 1.74 (95% CI: 1.54–1.97) [heterogeneity P < 0.001; I^2 80.5%] (Figure 5). When prospective effect estimates were replaced by cross-sectional effect estimates in the case of articles or populations providing both associations (meta-analysis 2; 16 studies; 85282 participants), the pooled OR for MetS for the highest levels of ferritin (vs. lowest levels) was 1.65 (95% CI: 1.41–1.93) [heterogeneity P < 0.001; I^2 79.2%] (Appendix Figure 1). The overall ORs for MetS components by high levels of ferritin (vs. lowest levels) (meta-analysis 3) are described in detail in Appendix Figures 2-6: high fasting glucose 1.69 (1.47–1.94) [heterogeneity P < 0.001; I^2 74.7%]; increased WC 1.52 (1.32–1.76) [heterogeneity P < 0.001; I^2 66.0%]; high triglycerides 1.96 (1.64–2.34) [heterogeneity P < 0.001; I^2 82.6%]; low HDL-C 1.45 (1.30–1.62) [heterogeneity P = 0.001; I^2 56.6%]; and high blood pressure 1.08 (0.98–1.18) [heterogeneity P = 0.010; I^2 46.7%].

Pooled ORs for MetS by sub-groups are shown in Table 11. The meta-regression analysis with study characteristics as independent variables (Table 11) showed that the pooled estimates for association between ferritin and MetS was stronger with RIA as the laboratory method for ferritin measurement than with other methods (Table 11) [meta-regression Exp(b) (95% CI): 0.89 (0.81–0.99), P = 0.033]. Adjustment for hepatic markers attenuated the association between ferritin and MetS [Exp(b) (95% CI): 0.77 (0.61–0.98), P = 0.036]. Higher cut-off points for upper category of ferritin levels reported in the studies (tertiles and quartiles of those values specific for sex/menopausal status according to the report) had a positive but statistically marginal significant effect on the pooled association between ferritin and MetS [Exp(b) (95% CI): 1.11 (0.98–1.26), P = 0.080]. Adjusting for BMI was associated with a marginal negative influence on the association between ferritin and MetS [Exp(b) (95% CI): 0.82 (0.65–1.04), P = 0.110]. When effect estimates from cross-sectional associations only were used, findings from meta-regression were in line with the above, with a clearer attenuating effect of adjusting for BMI [Exp(b)

(95% CI): 0.71 (0.52–0.96), P = 0.030], although the influence of the cut-off point for the upper category of ferritin levels was not observed (Appendix Table 21).

Table 11 Stratified odds ratio for metabolic syndrome in the studies (Meta-analysis 1)

Subgroup	Number of populations (studies)*	OR (95% CI)	I^2	Meta-regression P value
Study design				
Cross-sectional	22(12)	1.77(1.50-2.10)	70.8	
Prospective	11(5)	1.60(1.42-1.80)	0.0	0.490
Measure of association				
Odds ratio	28(14)	1.72(1.50-1.97)	67.0	
Hazard ratio	4(2)	1.68(1.41-2.01)	0.0	
Relative risk	1(1)	2.84(1.40-5.76)	--	0.425
Region				
Asia	19(11)	1.84 (1.54-2.19)	72.6	
Europe	11(5)	1.54 (1.36-1.75)	0.0	
America	3(1)	2.22 (1.60-3.09)	7.5	0.686
Adjusted for BMI				
No	15(9)	1.93(1.57-2.37)	75.6	
Yes	18(8)	1.61(1.44-1.81)	10.3	0.110
Adjusted for CRP				
No	18(10)	1.61(1.46-1.78)	0.0	
Yes	15(7)	1.90(1.53-2.36)	62.2	0.128
Adjusted for at least one inflammatory marker				
No	15 (9)	1.65(1.49-1.83)	0.0	
Yes	18 (8)	1.78 (1.45-2.18)	74.7	0.506
Adjusted for at least one hepatic function marker				
No	**21(11)**	**2.00(1.58-2.54)**	**79.2**	
Yes	**12(6)**	**1.60(1.46-1.76)**	**0.0**	**0.036**
Ferritin assay				
RIA	**10(4)**	**2.08(1.60-2.71)**	**76.3**	
QLA	**5(4)**	**1.60(1.30-1.97)**	**37.9**	
TIA	**9(5)**	**1.65(1.40-1.94)**	**13.8**	
Other	**9(4)**	**1.52(1.31-1.77)**	**0.0**	**0.033**
Sample size				
<1000	14(7)	1.56(1.35-1.80)	0.0	

Subgroup	Number of populations (studies)*	OR (95% CI)	I^2	Meta-regression P value
≥1000	1910)	1.80(1.53-2.12)	74.3	0.365
Risk of bias				
score < median	18(11)	1.86(1.55-2.23)	72.8	
score ≥ median	15(6)	1.62(1.41-1.85)	17.0	0.204
Mean/median ferritin levels (Sex/menopausal-specific tertiles)				
Tertile 1	12	1.70(1.28-2.27)	79.7	
Tertile 2	10	1.90(1.64-2.19)	22.9	
Tertile 3	11	1.60(1.38-1.85)	19.3	0.597
Mean/median ferritin levels (Sex/menopausal-specific quartiles)				
Quartile 1	7	1.50(1.25-1.80)	8.9	
Quartile 2	9	1.88(1.33-2.67)	79.6	
Quartile 3	9	1.98(176-2.23)	2.3	
Quartile 4	8	1.53(1.29-1.80)	18.8	0.867
Cut-off point reported for highest category of ferritin levels (Sex/menopausal-specific tertiles)				
Tertile 1	11	1.50(1.31-1.71)	0.0	
Tertile 2	10	1.98(1.50-2.60)	8.1	
Tertile 3	9	1.87(1.62-2.15)	9.5	0.103
Cut-off point reported for highest category of ferritin levels (Sex/menopausal-specific quartiles)				
Quartile 1	7	1.53(1.33-1.77)	0.0	
Quartile 2	8	1.51(1.26-1.80)	0.0	
Quartile 3	9	2.28(1.77-2.93)	73.6	
Quartile 4	6	1.73(1.46-2.04)	0.0	*0.080*

*** The first number describes sex/menopausal status groups from each study, and second number (in parenthesis) means number of studies.**

The meta-regression analysis also showed that the pooled estimates of association between ferritin and high blood pressure were attenuated in studies adjusting for BMI and hepatic function markers (Appendix Table 22). On the other hand, adjusting for CRP strengthened the association of ferritin with high triglycerides and high glucose (Appendix Table 22). In studies with higher risk of bias, high ferritin was more strongly associated with high triglycerides, WC, and blood pressure ($P < 0.039$) (Appendix Table 22). The ferritin-low HDL-C association was stronger in studies involving Asian populations than in those involving European and American populations ($P = 0.054$) (Appendix Table 22). In addition, higher cut-off points used to define high ferritin concentrations were more strongly associated with high triglycerides ($P = 0.019$ for quartiles/$P = 0.043$ for tertiles of those cut-off points) and high blood pressure ($P = 0.028$ for quartiles/$P = 0.043$ for tertiles of those cut-off points) (Appendix Table 22).

Findings from studies for the systematic review but not included in the meta-analysis

All articles described significantly higher levels of ferritin in cases with MetS. Iwanaga et al. (146) and Martinelli et al. (25) reported P values for the comparison of mean ferritin levels, adjusting for age and sex only in their cross-sectional studies. Meanwhile, Hamalainen et al. reported ferritin means standardised for age, sex, CRP, smoking, and physical activity in a cross-sectional study (135). These authors also investigated the longitudinal association between changes in ferritin levels and MetS and its components after 6.5 years of follow-up, adjusting for age and baseline smoking and values of CRP, ferritin, and BMI (113). In this study, the authors did not exclude cases with MetS or its components at baseline and compared the findings based on the presence or absence of the outcomes at baseline. There were significant increases in ferritin levels for men when MetS was present after follow-up and not at baseline of the study. The same was true for women regardless of the presence of MetS at baseline. In men, developing high triglycerides was associated with positive change in ferritin levels regardless of that MetS component being present at baseline. In women, developing high glucose was associated with positive change in ferritin levels but only when the MetS component was not present at baseline (113).

Sensitivity analyses

The exclusion of any single study did not substantially affect the pooled estimates for associations between ferritin, MetS, and its components. The pooled estimate for association with MetS ranged from 1.55 (1.35–1.78) to 1.82 (1.60–2.07) for the analysis of cross-sectional and prospective studies and from 1.64 (1.52–1.76) to 1.79 (1.57–2.03) for analysis of cross-sectional studies only, after excluding individual studies.

Pooled ORs (95% CI) for association between highest compared to lowest quantile/ category of ferritin and MetS components ranged as follows: HBP 1.04 (0.93–1.16) to 1.13 (1.04–1.23); low HDL-C 1.36 (1.25–1.48) to 1.49 (1.31–1.68); HFG 1.57 (1.39–1.76) to 1.75 (1.50–2.05); high WC 1.48 (1.30–1.69) to 1.57 (1.35–1.83); and HTG 1.81 (1.58–2.07) to 2.06 (1.69–2.51).

The significant findings and trends on sources of heterogeneity for the associations evaluated in general, persisted across the sensitivity analysis. In meta-analysis 1, the sensitivity analysis showed that adjusting for hepatic function markers or BMI remained significant attenuating factors of the ferritin-MetS association. Only the exclusion of the VIKING study slightly affected the significance of adjustment for BMI as a factor of influence in the meta-regression analysis ($P = 0.059$).

In meta-analysis 2, adjusting for hepatic function markers and ferritin assay retained its influence in the ferritin-MetS association after excluding any single study apart from the study of Li et al. ($P > 0.05$). The positive trend for cut-off values for high ferritin and strengthening of ferritin-MetS association was lost when six studies were individually removed from the meta- regression analysis ($P > 0.1$).

The strengthening of the association between ferritin and high blood pressure and triglycerides by quartiles of cut-off values reported for high ferritin persisted in the sensitivity analyses ($P < 0.05$ for high triglycerides, $P < 0.064$ for high blood pressure). The same was true when investigating the effect of adjusting for BMI as a factor of influence in the association between ferritin and high blood pressure ($P <$

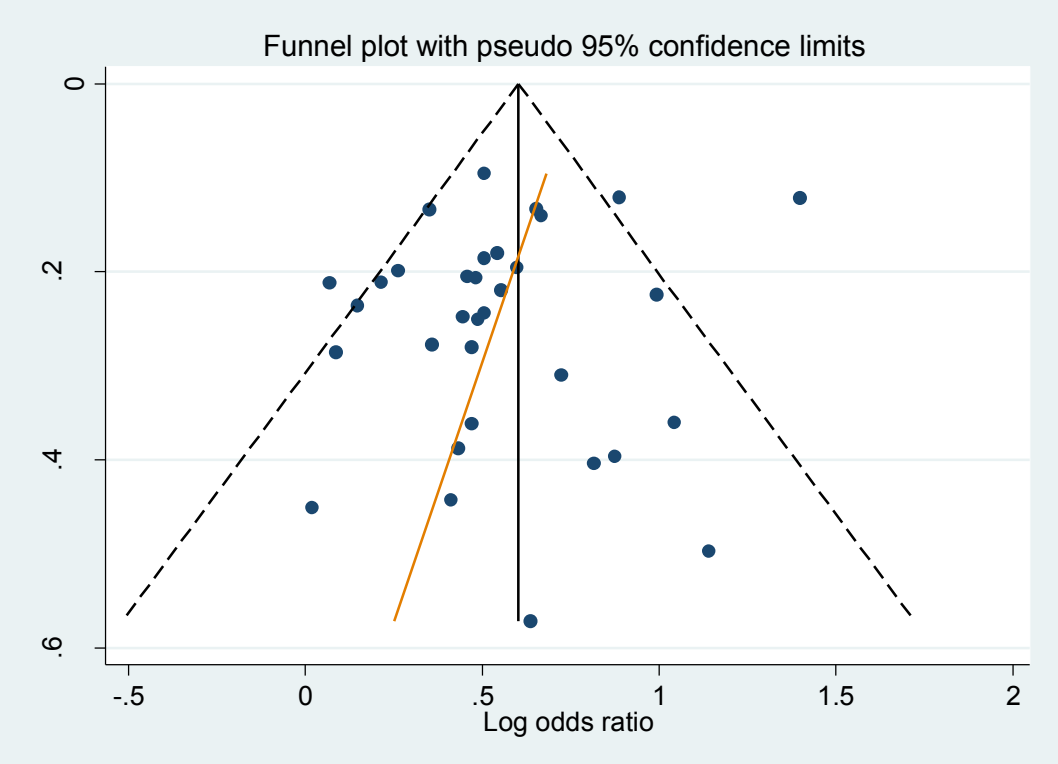
0.05). The effects of adjusting for CRP or other inflammatory markers and type of ferritin assay on the ferritin-high glucose association were not consistent across the sensitivity analysis. The individual exclusion of two studies attenuated the effect of world region in the association between ferritin and low HDL-C ($P > 0.1$).

Apart from the modifications described above, no other substantial changes in findings from meta-regression analysis were observed in sensitivity analyses.

Publication bias

The funnel plot for the ferritin-MetS association was asymmetrical with most of the studies located on the top left of the diagram (Figure 6). However, according to Begg's and Egger's tests, there was no evidence for publication bias ($P > 0.05$).

Figure 6 Funnel plot of the standard error of the natural logarithm of the effect estimate versus the natural logarithm of the risk ratio from each study.



Each point represents the association reported by sex/menopausal groups accordingly to figure 5. The red regression line highlights any asymmetry in the plot (in this case, the left of the diagram).

3.4 Discussion

There were contrasting findings in the association between ferritin and MetS or its components in cross-sectional analyses of two Scottish populations and the meta-analysis/systematic review. The meta-analysis suggested a positive overall association between ferritin and MetS but I found that the association was not independent of covariates in the VIKING study. Similarly, there was little or no evidence of association between ferritin and selected MetS components in the SHeS analysis after adjustment for covariates. The meta-regression for the ferritin-MetS association also identified weaker associations when the studies adjusted for BMI and hepatic function, as in the VIKING study and SHeS. Moreover, subgroup and meta-regression analyses also showed that in studies with higher cut-off points defining upper categories of ferritin levels, the association with MetS tended to be stronger. The association between ferritin and high triglycerides or blood pressure was markedly stronger in studies with higher cut-off points for high ferritin. In fact, cut-off points to define high ferritin in the VIKING study and SHeS were among the lowest ones reported. With regard to the overall association between ferritin and MetS components, stronger positive associations were observed with triglycerides and fasting glucose in comparison with other components.

Ferritin and MetS: Comparison with previous systematic review/meta-analyses

In the present meta-analysis, we describe a similar pooled overall positive OR for ferritin and MetS to that recently reported by Abril-Ulloa et al. (18), [(1.76 (95% CI: 1.57–1.97) vs. 1.73 (1.54–1.95) respectively]. However, the present meta-analysis had several differences from the previous one. First, the inclusion criteria of the present systematic review/meta-analysis required adjustment for at least age and sex. Second, there were four additional prospective studies (22, 105, 113, 145) and six additional cross-sectional studies (25, 136, 143-146). Third, I explored adjustment for BMI and hepatic function markers and threshold effects of ferritin values across study populations as sources of influence on the overall ferritin-MetS association. Lastly, associations between ferritin and individual MetS components were also described to identify whether there were any differences.

The work of Abril-Ulloa et al. was followed by another meta-analysis on ferritin and MetS in late 2015, with only eight studies selected between 2006 and 2014 and with no sub-group analysis and narrative synthesis (147). The authors reported a pooled OR (95% CI) of 1.20(0.69–1.71) for the association. The low statistical power in this study may have influenced the lack of a statistically significant association. I was unable to identify relevant differences in the inclusion/exclusion criteria to explain the low number of studies selected in comparison to the study of Abril-Ulloa et al. and the present meta-analysis.

Factors influencing the ferritin-MetS association

Neither Abril-Ulloa et al. (18) nor I found that study design, kind of effect estimate, geographic area, and study size influenced the ferritin-MetS association. The trend identified but not discussed by Abril-Ulloa et al. of a stronger association in studies which used immunoradiometric assays for ferritin measurement than in those which used other assays (P = 0.091) (18), was statistically significant (P = 0.044) in the present meta-analysis. In contrast to the study of Abril-Ulloa et al. (18), in this updated meta-analysis, adjustment for CRP levels was not identified as a source of heterogeneity for the ferritin-MetS association. A possible explanation is that Abril-Ulloa et al. (18) included some articles reporting unadjusted associations (23, 148, 149). I found that adjusting for CRP strengthened the pooled association with triglycerides, similar to the effect observed by Abril-Ulloa et al. for the ferritin-MetS association which was unexpected. CRP levels are considered a confounder since inflammation increases ferritin levels because ferritin is also a phase-acute reactant (9), and cardiometabolic risk has been widely associated with inflammatory response (72). One would expect effect estimates for ferritin-MetS or ferritin-triglycerides association to be attenuated in CRP-adjusted models rather than the pattern observed.

There were no differences in average ferritin levels or cut-off values for high ferritin by category of laboratory assay (data not shown). Therefore, the influence of the assay in the heterogeneity of ferritin-MetS association cannot be attributed to the threshold effect of the values of ferritin measurement. Since the meta-analysis by

Abril-Ulloa et al. also described a similar finding (18), possible explanations should be considered. However, there are no major differences in the accuracy of the current methods for measuring serum ferritin to explain the heterogeneity observed. The heterogeneity of the ferritin-MetS association by ferritin assay could also be a chance finding.

Adjusting for BMI and hepatic function markers (mostly transaminases) attenuated the pooled ferritin-MetS association across the studies evaluated. BMI is a well-known anthropometric predictor of CMD (150) and is positively correlated with iron stores (74). Obesity, estimated as high BMI, is also associated with both iron deficiency and increased ferritin. It appears that adipocytokines stimulate synthesis and secretion of the hormone hepcidin which inhibits intestinal iron absorption and release by tissues, causing iron deficiency (75). Similarly, low-grade inflammation in obesity can lead to increasing ferritin levels even in the context of iron deficiency (75). Iron excess in obesity could be explained by mechanisms of IR affecting iron homeostasis (151, 152). Thus, adjusting for BMI allows investigation of whether any ferritin-MetS association exists independently of obesity. More than half of the studies included did not adjust for BMI, and their authors did not give a rationale for not using BMI as covariate. Meanwhile, because ferritin is mostly produced in the liver, damage to hepatic cells positively influences circulating ferritin levels because it gets released into the bloodstream (73). In addition, pathological hepatic iron excess, such as in HH, can lead to liver injury and concomitant increased levels of transaminases (153). Similarly, hepatic function markers have been associated with cardiovascular risk factors (71) and CMD (154). In future research, the role of adjustment for BMI, hepatic function markers for evaluating confounding, effect modification, and potential underlying mechanisms should be considered.

Ferritin and MetS components

Stronger associations were observed between ferritin and high triglycerides or high fasting glucose than with other components of the MetS. There is growing experimental evidence that metabolism of glucose and of iron are interrelated and in a bidirectional way (9, 11). For instance, in murine models, starvation-induced

gluconeogenesis promoted iron hepatic deposition, and high hepatic stores of iron caused hyperinsulinemia by decreasing insulin extraction or affecting insulin signalling (9). This latter effect of iron could promote dyslipidaemia owing to high triglycerides. The association between ferritin and triglycerides could also be two-way based on findings in animals, where high-fat diets stimulated intrahepatic deposition of iron (9). In light of the above, high levels of glucose and triglycerides appear to be the components that make the largest contribution to a positive association between ferritin and MetS. The finding that the association between ferritin and MetS remained significant after adjustment for IR (HOMA-IR) in the four studies that included this adjustment is interesting. In two of these studies that showed unadjusted and adjusted associations, a marked attenuation of the association was observed only in one (OR (95%CI) 3.45 (3.03–3.92) to 1.99 (1.70–2.33)) (133). The above points imply that association between ferritin and MetS is not entirely explained by the associations with hyperinsulinemia and that there are alternative and still unknown, underlying mechanisms.

The subgroup analysis of the association of ferritin and MetS components suggested the presence of heterogeneity between the studies, some of which were similar to those described for the ferritin-MetS association. For instance, the high blood pressure-ferritin association was weaker when the studies adjusted for BMI and hepatic function markers as was found for the association with MetS. The associations between ferritin and high triglyceride and high blood pressure were significantly influenced by the cut-off value for high ferritin reported in the studies, as found in the association with MetS (meta-analysis 2). On the other hand, there were other sources of influence specific to individual associations between ferritin and other MetS components. The isolated finding of stronger association between ferritin and low HDL-C in studies from Asia seems to be related to a meta-analysis of 23 studies from the Asia-Pacific region on prevalence of cardiovascular risk factors. This meta-analysis highlighted that prevalence of the low HDL-C phenotype was higher in Asian populations in comparison with populations from Australia and New Zealand (155). The reason behind this difference in prevalence is still not clear but iron status could be related to a complex interaction of nutritional and genetic

factors that affect the phenotype (155). Meanwhile, studies with greater risk of bias can overestimate specific associations between ferritin and increased WC, triglycerides, and blood pressure on the basis of low representativeness and/or non-adjustment for BMI. It is unclear why these factors were not similarly found as sources of influence in the ferritin-MetS association. The above finding suggests that each component of MetS may have specific patterns of association with ferritin regardless of the pattern with the risk cluster.

Interpretation of findings from the Scottish studies in the context of key findings from the meta-analysis

There were no independent associations between ferritin and MetS or its components or between ferritin and IR in the VIKING study. In the SHeS, there were only associations between increasing ferritin and low HDL-C in postmenopausal women and with high WC in men. Although the meta-analysis identified a pooled positive significant association between ferritin and MetS, our negative findings in the Scottish studies are similar to those of several studies included in the meta-analysis. Zelberg et al. did not find a significant association in an Israeli population, Kilani et al., in men or premenopausal women. Interestingly, along with the latter study, the studies by Jehn et al., Kim et al., Lee et al., and Shi et al. failed to find an independent association in men, a demographic subgroup with higher iron status. There were no consistent associations by sex or menopausal status, with some studies reporting associations in women but not in men and others reporting the reverse.

The non-significant associations in the Scottish studies could be explained if there is a threshold effect of ferritin concentrations on the association with MetS. and more consistently with high triglycerides and blood pressure. The findings of the meta-regression showed a strengthening of the ferritin-MetS association when higher cut-off points were used to define the highest ferritin levels. This apparent threshold effect was also seen for the pooled associations of serum ferritin with high triglycerides and blood pressure. The Scottish studies were located in the lowest sex/menopausal-specific tertiles and quartiles for those ferritin cut-off points, so it is possible that ferritin levels were too low to trigger a significant independent association with MetS or several of its components. Excluding people with anaemia

or iron deficiency from the Scottish population did not affect the finding that ferritin levels were lower than those in other studies.

Another reason for the general lack of independent associations in the Scottish studies could be, at least partially, related to high prevalence of confounding factors. BMI values affected the associations found in men and postmenopausal women in the VIKING study and SHeS. More than half of the individuals in both Scottish studies were overweight or obese, with the prevalence of overweight and obesity being 70% and 24%, respectively, in the VIKING study. Moreover, Scotland has the second highest prevalence of obesity among the members of the Organisation for Economic Co-operation and Development after the United States of America (156), reaching figures of 22% and 24% of Scottish men and women, respectively, in 2003 (156), in line with the 24% obesity prevalence in the VIKING study (2013–2015). The association between ferritin levels and cardiometabolic risk markers could differ between populations of different countries owing to genetic and/or ethnic differences influencing the amount of body iron and interaction with other dietary and non-dietary factors. Given this complex matrix of influences, differences in distribution of confounding factors and the strength of the associations of these with iron and outcome variables are likely to exist.

Inverse associations in SHeS and VIKING

Ferritin levels and high blood pressure showed a U-shaped trend in their association among premenopausal women from the SHeS. This finding might be plausible since both low and high iron status may be related to cardiometabolic risk, although potential theoretical mechanisms for iron deficiency are still unclear. I did not find studies reporting a similar association, but iron deficient compared to non-deficient subjects have shown higher pulmonary blood pressure (157). In contrast, a recent study did not find difference in the prevalence of pulmonary hypertension by low and normal iron status in African-American individuals (158). The ferritin-high blood pressure association in premenopausal women could be due to chance, since there were no other inverse or U-shaped associations between ferritin and other MetS components in adjusted analyses.

The association between ferritin and HbA1C was the only cardiometabolic risk outcome independently associated with ferritin in the VIKING study. However, this relationship was unexpectedly inverse and taking into account the absence of association between ferritin and other cardiometabolic risk markers, this could also be a chance finding. In addition, the inverse association might be spurious in terms of increased HbA1c in individuals with iron deficiency due to an increase in the erythrocyte lifespan. This spurious increase in HbA1c with iron deficiency is not accompanied by concomitant rise in glucose indices (111), which is coherent with the pattern found in the VIKING study.

Strengths and limitations

To the best of my knowledge, this study appears to be first meta-analysis on ferritin, MetS, and its individual components. This analysis has also described for first time the association between ferritin and MetS in Scottish or British populations. In addition, the investigation of the influence of adjustments for body mass and hepatic function and of threshold effects of ferritin on the ferritin-MetS association across the studies represents another novel contribution. On the other hand, some findings from the subgroup and meta-regression analysis were not consistent throughout the sensitivity analysis. This implies limitations in statistical power or chance findings arising from multiple testing. Given the different assumptions in the calculation of effect estimates from prospective and cross-sectional studies, analysing them together might not be appropriate, although no heterogeneity by effect estimate or study design was detected in the subgroup meta-regression analysis. However, this potential limitation was balanced by conducting an additional meta-analysis specific to cross-sectional studies with all the studies reporting associations as ORs.

In conclusion, the meta-analysis suggests a significant overall positive association between ferritin and MetS but this association was not observed after adjustment for a variety of factors in the two Scottish populations described in the present chapter. Hepatic function, BMI, and type of ferritin assay appear to influence the ferritin-MetS association. Threshold effects of high ferritin concentration on the associations

with MetS, high triglycerides and blood pressure, and different distributions of confounding factors may explain the lack of independent association between ferritin and MetS or its components in the Scottish studies. High triglycerides and glucose are the MetS components most strongly associated with ferritin levels and could explain most of the association with the risk cluster known as MetS. The next chapter describes the association between sTfR levels and MetS or insulin resistance in Croatian adults.

Chapter 4

Soluble transferrin receptor levels and its association with metabolic syndrome and insulin resistance in Croatian adults

4.1 Introduction

There are other important proteins in iron metabolism in addition to ferritin, such as transferrin and TfR (159, 160). TfRs are of particular interest to researchers since they act as a sensor for body iron demands. Iron transport in the plasma is carried out by transferrin, which donates iron to the cells through its interaction with a specific membrane receptor, the TfR. A soluble form of the TfR has been identified in human serum. Soluble TfR (sTfR) is a truncated monomer of the tissue receptor, lacking its first 100 amino acids, which circulates in the form of a complex of transferrin and its receptor. The higher the body iron stores, the lower the TfR levels in cell membranes which in turn down-regulates intestinal iron absorption (161). Increased levels (> 5 and 4.4 mg/L in men and women respectively) of TfRs in cell membranes reflect body iron deprivation, and intestinal iron absorption is up-regulated as a compensatory mechanism. Levels of sTfR are proportional to tissue concentrations (162). sTfR and ferritin levels are influenced by the acute phase response of inflammation, so their measurement should include additional measurements of inflammatory markers (163).

The association between sTfR and cardiometabolic risk factors has not been widely investigated. A recent meta-analysis of eight studies reported conflicting results for the association between sTfR and T2D and described the limited power of the included studies owing to small sample sizes (19). Only one study described the association between sTfR and MetS (135) but did not adjust for BMI, few studies have evaluated the association between sTfR and some MetS components (164, 165), and concomitant examination of the association between sTfR and IR has not been conducted. Most of these studies reported no evidence of an association between

sTfR and MetS and very few reported associations with MetS components. The primary aim of this thesis chapter was to investigate whether sTfR levels were associated with MetS, its components, IR, and HbA1C in a relatively large and well-characterised population. The secondary aim was to describe the association between ferritin and the outcomes mentioned above.

4.2 Material and Methods

4.2.1 Participants

The participants were identified from the 10,001 Dalmatians research programme, namely the sub-cohort recruited from the island of Vis in Croatia. A total of 1,029 subjects were initially recruited, aged 18–93 years, from the villages of Vis and Komiza during 2003 and 2004 within a larger genetic epidemiology study (166-168). In the original study, the only eligibility criterion was age of 18 years or above. sTfR and ferritin levels were measured in 774 subjects, of whom 49 subjects with missing values for covariates (age, fibrinogen, glycosylated haemoglobin, smoking and alcohol consumption, BMI, and history of CMD) were excluded, resulting in a study population of 725 people. Distribution of exposure, adjustment variables, and outcomes were similar among those with and without missing data with the exception of fibrinogen levels, which were higher in those with complete data. Ethical approval was issued by the Multi-centre Research Ethics Committee for Scotland (MREC) under designation MREC 01/0/71, and all participants gave written informed consent.

4.2.2 Clinical and anthropometric measurements

Anthropometric measurements included height measured using a stadiometer, weight, and WC (measured half-way between the lowest rib and iliac crest). Blood pressure was measured in a seated position, after at least five minutes of rest. Two measurements were made, and only the second one was recorded, in order to reduce the “white coat” effect. Menopausal status was confirmed on the basis of self-report with pre-menopausal status defined as continuing menstruation and post-menopausal status, absence of menstruation. Physical activities at work and at leisure were self-reported on a four-point rating scale (sitting, light, moderate, and hard).

4.2.3 Biochemical measurements

Blood samples were taken after overnight fasting. Classical biochemical analyses of the blood sample included the following: triglycerides, using UV photometry with glycerolphosphate-oxidase (GPO PAP) (Olympus kit OSR60118); HDL cholesterol, using homogeneous enzyme method with modified polyethylene glycol and cyclohexane-sulphate (Olympus kit OSR6195); glucose, using UV hexokinase photometry; and HbA1C (whole-blood sample), using cation exchange, immunochemistry electrophoresis, and affinity linking, compatible with the DCCT/UK PDS standard. The measurements of HbA1C and glucose were performed using Olympus kit OSR6192, OSR6121, and OSR6221. Manufacturer's reagents were used with their internal quality control (ODC003 or ODC004 for glucose and ODC022 for HbA1C). Fibrinogen was measured by the Clauss method using an MDA 180 coagulometer (Biomerieux, Marcy l'Etoile, France) with reagents from the manufacturer. The calibrant used was the 8th British Standard (NIBSC). Electrochemiluminescence immunoassay (Roche) was used to measure ferritin and Tina-quant® immunoturbidimetry (Roche), to measure sTfR. The laboratory was ISO accredited and daily internal controls were performed (calibrator 66300). Repeated measurements of the sub-set of samples provided very high agreement (kappa 0.92 for the lowest pair of estimates). The coefficient of variation was less than 5% for each biochemical measurement. The biochemical assays were conducted by using an OLYMPUS AU400 chemistry immunoanalyzer. IR was estimated by the homoeostatic model of assessment using the following formula: glucose levels [mmol/L] × insulin mU/L/22.5 (59).

4.2.4 Metabolic syndrome

The following cut-off points from the international consensus definition for MetS were used (40): triglycerides ≥1.7 mmol/L, HDL-C <1.0 mmol/L in men and <1.3 in women, glucose ≥5.6 mmol/L (or drug treatment for elevated glucose), SBP ≥130 mm/Hg and/or DBP ≥85 mm/Hg (or antihypertensive drug treatment), and WC ≥94 cm in men and ≥80 cm in women. Information on lipid-lowering medications was

not available to complement the component of high triglycerides. MetS was defined as the presence of three or more variables meeting the definitions above.

4.2.5 Data analysis

The analyses were stratified by sex/menopausal status and by adjusting for sex/menopausal status in analyses of the whole cohort. All continuous study variables were summarised as median (interquartile ranges) by sex/menopausal status and differences were tested using the Mann-Whitney *U* test. Logistic regression models were used to describe the associations between sTfR and ferritin as exposure variables, with MetS and its components as outcome variables. sTfR and ferritin were used as continuous variables in terms of standard deviation units of their log-transformed levels (Z scores or standardised values) to facilitate interpretation of ORs. ORs of each outcome are therefore described for each standard deviation in log-transformed iron marker. Unlike the analyses in chapter 3, in this analysis the iron markers were not additionally treated as categorical variables of quantiles because the pre-menopausal women group was small and there were small numbers by categories of ferritin in preliminary explorations of the dataset, e.g. there were no cases of high triglycerides in the highest tertile of ferritin. Multivariable models with age, levels of fibrinogen, smoking (never smoker, ex-smoker, and current smoker), alcohol consumption (no/yes), and BMI as covariates were used to investigate whether the associations were independent of these potential confounding factors. This set of confounding factors was chosen on the basis of possible influence of acute phase or subclinical inflammation in terms of fibrinogen levels (169, 170) and general adiposity on levels of iron markers and/or outcome variables. Relationships between measures of iron status and IR and HbA1C were described using multiple linear regression analyses adjusting for potential confounders as listed above with additional adjustment for treatment with insulin and/or hypoglycaemic drugs (yes/no). The normality of distributions was assessed using histograms and Kolmogorov-Smirnov tests. For the linear regressions, transformed values of skewed variables were used as follows: logarithm of sTfR, ferritin, HOMA-IR values, BMI and fibrinogen values, square of age, and square root of glycosylated haemoglobin. The above set of arithmetic functions allowed the best approximation to the normal

distribution for each variable. Self-reported CVD (heart attack, stroke) and diabetes and self-reported physical activities at work and at leisure (sitting [reference], light, moderate, and hard) were additionally used as covariates in sensitivity analyses. In order to avoid collinearity, treatment with insulin and/or hypoglycaemic drugs (yes/no) was not considered in the multivariable model when diabetes was used as covariate. As in Chapter 3, I conducted sensitivity analyses on the basis of exclusion of individuals with anaemia, very high ferritin (>300 μg/L), and potential inflammation/infection (fibrinogen > 4.7 g/L). A P value <0.05 was considered statistically significant. Data were analysed using Stata version 11.0 (StataCorp).

4.3 Results

There were modest differences between the included and excluded subjects.. The group of excluded individuals had a higher proportion of men and lower fibrinogen concentrations than the people that were included (Appendix Table 23).

The study involved a total of 725 subjects, stratified in 3 groups by sex/menopausal status (Table 12). Men had higher values of ferritin than women, while postmenopausal women had higher values of ferritin than premenopausal women. Postmenopausal women had significantly higher levels of sTfR than men, while the comparison with pre-menopausal women yielded insignificant results (Table 12). HOMA-IR was higher among men and postmenopausal women than among pre-menopausal women with a similar pattern observed for MetS components. Prevalence of high WC was above 60% in all groups (Table 12) and prevalence of MetS was above 50% in postmenopausal women and men. Prevalence of MetS was higher among post-menopausal women than men and pre-menopausal women (Table 12). There was an inverse correlation between sTfR and ferritin levels (log-transformed values of both markers) ($r = -0.396$, $P < 0.001$).

sTfR levels (median and interquartile range) were similar for people with and without MetS in each sex and menopausal status group:
Premenopausal women MetS 3.19(2.71-3.72) v. No MetS 3.35(2.66-4.48) μg/L, P=0.426;

Postmenopausal women MetS 3.09(2.62-3.65) v. No MetS 3.17(2.73-3.91) µg/L, P=0.825;
Men MetS 2.99(2.56-3.76) v. No MetS 3.09(2.62-3.65) µg/L, P= 0.828.

Ferritin levels (median and interquartile range) were higher in subjects with MetS in postmenopausal women and men:
Premenopausal women MetS 31.6(9.9-59.7) v. No MetS 24.2(13.7-40) µg/L,
P= 0.319;
Postmenopausal women MetS 69.5(42-106.1) v. No MetS 38.2(25.4-73.2) µg/L, P=0.002;
Men MetS 176.7(108.3-305.3) µg/L v. No MetS 121(73-178.5) µg/L, P<0.001.

There was no statistically significant association between standardised values of sTfR and MetS (Table 13) and its components (Appendix Table 24) in any of the sex/menopausal status groups or in the whole sample (Appendix Table 25). Since the high prevalence of MetS and some components could limit the power of the study to detect an association with sTfR, I additionally conducted linear regression analysis between log-sTfR and log-transformed values of WC, HDL-C, glucose, triglycerides, SBP, and DBP. The adjusted linear regressions did not show significant relationships (Appendix Table 26). Further adjustment for treatment with insulin and/or hypoglycaemic drugs in associations with glucose, and for anti-hypertensive medication in associations with SBP and DBP, did not alter the significance of the above findings.

Table 12 Distribution of iron status and cardiometabolic risk by sex and menopausal status

	Pre-menopausal women	Post-menopausal women	Men	P *values*		
				Premenopausal vs postmenopausal women	Men vs. premenopausal women	Men vs. postmenopausal women
n	151	290	284			
Age (years)	40 (33-47)	67(57-74)	57 (46-68.7)	< 0.001	< 0.001	< 0.001
BMI (kg/m^2)	26.6(21.6-27)	28.3(25.4-30.9)	27.6(25.2-29.6)	< 0.001	< 0.001	0.013
Ferritin (µg/L)	25.5(12-45.9)	65.9 (38.6-102.2)	141.3 (90.5-233.7)	< 0.001	< 0.001	< 0.001
sTfR (mg/L)	3.19(2.63-4.05)	3.17 (2.73-3.90)	3.07 (2.62-3.68)	0.758	0.069	0.038
Glucose (mmol/L)	5.0(4.6-5.4)	5.6(5.0-6.2)	5.5(5.0-6.1)	< 0.001	< 0.001	0.504
TG (mmol/L)	1.2(0.9-1.5)	1.5(1.1-2.0)	1.5(1.1-2.3)	< 0.001	< 0.001	0.542
HDL-C (mmol/L)	1.16(1.09-1.25)	1.14(0.98-1.23)	1.13(0.96-1.22)	0.011	0.001	0.410
SBP (mmHg)	118(108-128)	145(130-161)	138(123-150)	< 0.001	< 0.001	< 0.001
DBP (mmHg)	75(68-80)	81(75-89)	81(75-89)	< 0.001	< 0.001	0.820
WC (cm)	83(76.9-91.5)	99.2(91.7-105.4)	98.3(92-105.1)	< 0.001	< 0.001	0.403
Insulin mU/L	5.0(4.0-8.0)	7.0(5.0-11)	6.0(4.0-9.0)	< 0.001	< 0.001	0.147
HOMA-IR	1.17(0.78-1.89)	1.69(1.06-2.93)	1.58(1.01-2.60)	< 0.001	< 0.001	0.141
HbA1C (%)	5.1(4.9-5.4)	5.5(5.2-5.7)	5.2(5.0-5.6)	< 0.001	< 0.001	< 0.001
Fibrinogen (g/L)	3.5(2.9-4.0)	3.9(3.4-4.5)	3.5(2.9-4.1)	< 0.001	0.714	< 0.001
MetS and its components n(%)						
High blood pressure¶	43(28.3)	237(81.7)	198(69.7)	< 0.001	< 0.001	0.001
High glucose †	27(17.8)	148(51.0)	131(46.1)	< 0.001	< 0.001	0.208
Low HDL-C	137(90.7)	277(95.5)	82(28.9)	0.040	<0.001	<0.001
High triglycerides	25 (16.6)	118(40.7)	117(41.2)	< 0.001	< 0.001	0.902
High WC	94 (62.3)	278(95.9)	191(67.3)	< 0.001	0.296	< 0.001
MetS	51(33.8)	257(88.6)	144(50.7)	< 0.001	0.001	<0.001
Smoking n (%)						
Yes	64(42.4)	47(16.2)	85(29.9)			
No	58(38.4)	184(63.4)	73(25.7)			
Ex-smoker	29(19.2)	59(20.3)	126(44.4)	< 0.001	< 0.001	< 0.001
Alcohol consumption n (%)	79(52.3)	108(37.2)	230(81)	0.002	< 0.001	< 0.001
Cardiovascular disease n (%)	12(7.9)	150(51.7)	102(35.9)	< 0.001	< 0.001	< 0.001
Diabetes n (%)	3(2.0)	27(9.3)	20(7.0)	0.002	0.017	0.201

Data are median (interquartile range) or n (%). Comparison between groups by Mann-Whitney U and χ^2 test. BMI, body mass index. sTfR, soluble transferrin receptor. TG, triglycerides. SBP, systolic blood pressure. DBP, diastolic blood pressure. WC, waist circumference. HDL-C, HDL cholesterol. MetS, metabolic syndrome. HOMA-IR, homeostatic model assessment insulin resistance. † Includes additionally individuals who reported current use of oral hypoglycemic medications or insulin regardless of fasting glucose values.¶ Includes additionally individuals who reported current use of antihypertensive medications regardless of blood pressure values.

Table 13 Odds ratios (95% CI) for metabolic syndrome per sex/menopausal-specific SD of the iron markers in the study subjects categorised by sex and menopausal status

	Z score log-sTfR		**Z score log-ferritin**	
	Non-adjusted	Adjusted*	Non-adjusted	Adjusted*
Premenopausal women				
MetS	1.32 (0.90-1.92)	1.35 (0.90-2.02)	1.13 (0.80-1.59)	1.35 (0.90-2.02)
Postmenopausal women				
MetS	0.99 (0.69-1.43)	0.73 (0.47-1.15)	**1.65 (1.17-2.31)**	**1.71 (1.12-2.62)**
Men				
MetS	1.05 (0.83-1.33)	0.87 (0.66-1.17)	**1.90 (1.44-2.50)**	**1.78 (1.31-2.42)**

***Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes) and BMI. TG, triglycerides. BP, blood pressure.. WC, waist circumference. HDL-C, HDL cholesterol. MetS, metabolic syndrome. Significant associations are show in bold (P<0.05).**

Standardised values of ferritin were significantly associated with MetS in men and postmenopausal women (Table 13). Ferritin was also significantly associated with higher odds of having MetS components (except high blood pressure) in men, in unadjusted models and models adjusted for age, fibrinogen levels, alcohol intake, and smoking (Appendix Table 24). In the whole sample, the adjusted associations found for ferritin and MetS and its components were similar to those reported in men (Appendix Table 25).

In a separate analysis for women adjusting for menopausal status, sTfR was not associated with MetS or its components, and ferritin was independently associated with high triglycerides and MetS (Appendix Table 27).

sTfR levels were positively associated with IR in postmenopausal women and men: postmenopausal women (Beta = 0.34 [0.05 to 0.63], P = 0.020) and men (Beta = 0.44 [0.14 to 0.75], P = 0.004) (Appendix Table 28). In the whole sample, sTfR levels were associated with IR ($P < 0.05$), but no association was observed for ferritin in similar analyses (Appendix Table 28). The relationship between sTfR and HOMA-IR was driven by the relationship between sTfR and insulin levels, which was similarly significant (Appendix Table 28), whereas adjusted association with glucose levels was not statistically significant (Appendix Table 26).

In the unadjusted analysis, HbA1C, a marker of longer-term glucose metabolism, was significantly associated with sTfR in men and with ferritin in the whole sample, but after adjustments, there were no significant independent associations (Appendix Table 29).

I additionally used diabetes, cardiovascular disease and categories of physical activity as covariates in the adjustment models, but the estimates of the associations described above between the iron markers, MetS, IR, and HbA1C and the statistical significance did not change substantially.

In sensitivity analyses, the exclusion of subjects with very high ferritin (n=65) attenuated all of the significant associations previously described, presumably partly due to the lower sample size (ORs decreased and P>0.05), with exception of the ferritin-MetS association in postmenopausal women, whose OR and 95%CI remained similar [adjusted OR (95%CI) 1.64(1.05-2.54), P=0.027]. However, the statistical significance of this association was attenuated after removal of individuals with iron deficiency (n=66) [adjusted OR (95%CI) 1.71(0.93-3.14), P=0.080], as well as the ferritin-high WC association described in men [adjusted OR (95%CI) 1.43(0.91-2.24), P=0.115]. Exclusion of subjects with potential inflammation and/or infection (high fibrinogen levels) (n=99) attenuated the ferritin-high glucose and ferritin-high WC associations in men. On the other hand, the previous non-significant association between serum ferritin and high triglycerides in postmenopausal women reached statistical significance [adjusted OR (95%CI) 1.37(1.02-1.85) P=0.035]. No other substantial modifications were observed in sensitivity analyses for significant or non-significant association described in the original sample.

4.4 Discussion

In this study of a population with high prevalence of MetS, sTfR levels were associated with IR but not with MetS. Conversely, serum ferritin was statistically significantly associated with MetS. These findings suggest that different iron-related proteins are involved in cardiometabolic risk by separate underlying mechanisms.

The lack of association between sTfR levels and MetS is consistent with findings in studies of different sizes ranging from 155 to 1,969 subjects (Table 14). A Finnish study involving middle-aged subjects from the general population also found no significant association when controlling for confounding factors (Table 14) (135). In the same study, levels of sTfR levels were significantly higher in individuals with high WC (compared to normal WC) in adjusted analyses (135). A small study reported lower levels of sTfR in subjects with MetS in a sex-stratified analysis, but no additional adjustments were conducted (149) (Table 14). A significant positive age-sex-adjusted correlation of sTfR with WC but no evidence of a relationship with HDL-C and triglyceride levels in participants from the EPIC-Postdam study has also been reported (164) (Table 14). DBP and triglycerides increased across quartiles of sTfR but no association was found with WC, LDL-C, HDL-C, SBP, and fasting glucose levels in 1,262 women after adjustment for covariates (165) (Table 14). Different adjustments, statistical approaches, and discrepancies in methods measuring sTfR concentrations could contribute to the heterogeneity of results from different studies. Various commercial sTfR assays give disparate values because of the lack of an international standard. For instance, Hamalainen et al. reported similar sTfR levels (mean 2.9 mg/L) and prevalence of MetS (48% in men and 52% in women) to those we reported (135). Very high sTfR levels (mean 9.09 mg/L) with lower prevalence of some MetS components (high WC, 31%, low HDL, 43%) were reported by Aderibigbe et al. (165). Meanwhile, Montonen et al. reported lower median sTfR levels across ferritin quintiles between 1.0 and 1.9 mg/L (164). In addition, it is important to note that the above studies included diverse populations in Europe and South Africa which may have influenced the association patterns, sTfR levels, and prevalence of MetS components.

*Table 14 Studies in general population on sTfR and metabolic syndrome**

Authors, year of publication (reference)	Study	Location/ Universe	Study/Year of survey	Age range or mean (SD) (years)	Male (%)	Total sample	Cardiovascular risk markers (outcomes and analysis-effect estimate)	sTfR: Continuous /categorical-ordinal	Association		Adjustments
									Yes	No	
Montonen et al., 2012 (164)	Cross-sectional	Germany/ Potsdam population	Epic –Potsdam/ 1994-1998	35-65	37.9	1969	WC, HDL-C and TG (continuous). Partial Pearson Correlations	Continuous	With WC (r=0.13), (p<0.001).	With HDL-C and TG	Age and gender
Aderibigbe et al., 2011 (165)	Cross-sectional	South Africa/ Population from North West province	PURE/ 2005	≥35	0	1262	WC, LDL-C, HDL-C, TG, DBP, SBP and FG. Levels (mean and 95%CI) across categories of iron markers.	Categorical. Quartiles.	With TG: Higher levels in quartiles 3 and 4 vs quartile 1. With DBP: Higher in quartile 4 vs quartiles 1 and 2.	With LDL-C, HDL-C, WC, SBP and FG.	Age, BMI, smoking, alcohol consumption and CRP.
Leiva et al., 2013 (149)	Cross-sectional	Chile/Population from Talca.	Research Program of RiskFactors for Cardiovascular Disease of Talca, (PIFRECV)/2005	45-65	30.9	155	Metabolic syndrome(NCEP ATP-III) (dichotomic variable)	Continuous : means by Metabolic syndrome (yes/no)	Lower levels of sTfR in subjects with MetS	----------	Sex

Authors, year of publication (reference)	Study	Location/ Universe	Study/Year of survey	Age range or mean (SD) (years)	Male (%)	Total sample	Cardiovascular risk markers (outcomes and analysis-effect estimate)	sTfR: Continuous /categorical-ordinal	Association		Adjustments
									Yes	No	
Hamalainen et al.,2012 (135)	Cross-sectional	Finland/Middle –aged subjects from Pieksamaki who were born in 1942,1947,1952, 1957 or 1962	NP/2003-2004	52.1 ± 6.2 years(men) and 52.1 ± 6.2 years (women)	44.5	766	Metabolic syndrome(NCEP ATP-III) and its components (dichotomic variables)	Continuous : standardized means by categories of Metabolic syndrome and its components	Higher sTfR in subjects with increased WC	Metabolic syndrome, and high blood pressure, FG, TG and low HDL-C	Age, sex, hs-CRP, smoking and physical activity

NP, Not provided. sTfR, soluble transferrin receptor. WC, waist circumference. LDL-C, LDL cholesterol. HDL-C, HDL cholesterol. TG, triglycerides. DBP, diastolic blood pressure. SBP, systolic blood pressure. FG, fasting glucose. * Studies found in PUBMED and EMBASE by applying the searching "metabolic syndrome OR blood pressure OR fasting glucose OR waist circumference OR triglycerides OR HDL cholesterol" AND "transferrin receptor" (until July/2015). [Only studies in adults were included and in vitro studies, genetic studies and those conducted in specific populations (pregnant women, and patients with diseases) were not included]"

The finding of a positive association between sTfR and IR in postmenopausal women and men in this chapter is consistent with the unadjusted findings of Fernandez-Real et al. (171) and Huth et al. (172). Fernandez-Real et al. described an inverse association between sTfR levels and insulin sensitivity estimated by minimal modelling in 221 Spanish individuals (97 non-obese with normal glucose tolerance, 36 with impaired glucose tolerance, and 88 with T2D) (171). There was no evidence of association between sTfR and fasting glucose or insulin but positive correlation between sTfR with values of glucose and insulin during an oral glucose tolerance test was reported (171). I reported similar findings to Huth et al. who found that sTfR levels were significantly and positively correlated to HOMA-IR in 2,893 participants of the population-based Cooperative Health Research in the Region of Augsburg (KORA) F4 study (Germany) (172). In contrast, Arija et al. did not find significant correlation between sTfR and HOMA-IR, adjusting for sex, age, and BMI in Spanish non-diabetic individuals (n = 302), as observed in group of non-diabetic subjects who later developed diabetes (n = 153) (173). The relationship between sTfR and glucose metabolism might be easier to identify in the postprandial than in fasting state since Fernandez-Real reported a correlation with glucose concentrations after an oral glucose test tolerance test (171). In addition, an insulin-sensitising intervention of dietary change combined with exercise was associated with a decrease in sTfR levels in obese individuals (171). I found that additional adjustment for physical activity did not substantially affect the non-significant associations of sTfR with MetS and its components, but the measurement of physical activity is imperfect. In terms of genetic factors, a significant association between presence of transferrin receptor-1 gene *(TRFC)* polymorphisms (rs3817672, 210AG, S142G) and T2D has been described (174). In addition, individuals with 210A--G polymorphism showed higher sTfR levels, which correlated positively with glucose levels whereas in non-carriers, there was no relationship between those markers (174). These associations with polymorphisms were not confirmed in genetic consortia databases (175). However, other studies found other SNPs linked to both T2D and sTfRs. For instance, significant associations have been observed for loci in *TPMRSS6* with sTfR

($P = 3.47 \times 10^{-6}$) and T2D risk (176). These findings imply that a common third factor influences both circulating sTfR levels and diabetes susceptibility.

Although the finding of a positive association between sTfR and IR suggests that low iron status, in terms of high sTfR, is associated with cardiometabolic risk, this is not consistent with the absence of a concomitant association with MetS or its components in this cross-sectional study. A potential explanation is the effect of insulin on sTfR levels. Insulin upregulates erythropoiesis (177), of which sTfR is a surrogate. sTfR represents a valuable quantitative assay of marrow erythropoietic activity as well as a marker of tissue iron deficiency (178). Marrow erythropoietic activity appears to be the most important determinant of sTfR levels, causing variations up to 8 times below and up to 20 times above average normal values (178). The erythroblasts rather than reticulocytes are the main source of serum sTfR, whose levels decrease when erythropoietic activity is low and increase in situations of haemolysis or ineffective erythropoiesis (178). As insulin has been described to upregulate erythropoiesis (177), bone marrow could still be sensitive to insulin in hyperinsulinemia despite peripheral IR in the liver or muscle (the classical insulin sensitive tissues). If this is the case, sTfR might appear to be spuriously elevated and would not reflect the insulin sensitivity in other tissues. In addition, up-regulation in the expression of TfRs by insulin via a hypoxia inducible factor, as observed in human hepatic cells (HepG2) (179), is an alternative explanation.

There were differences by sex and menopausal status in the relationship of ferritin with MetS and its components since significant associations were found in men and postmenopausal women, but not in premenopausal women in our study. Theoretically, in men and postmenopausal women, the relationship between ferritin and cardiometabolic risk might be more obvious owing to higher iron accumulation than in women who lose iron during menstruation. However, as observed and discussed in the meta-analysis in chapter 3, there were studies reporting significant association between ferritin and MetS in pre-menopausal women but not in men. Similarly, the ferritin-MetS association may not be related to the potential threshold effect of ferritin concentration mentioned in the previous chapter, since high ferritin

concentration (the cut-point at 75th percentile) in men and postmenopausal women of Croatia-Vis study fit in the middle range of cut-points reported for increased ferritin of the studies reported. In addition, since effect estimates (unadjusted and adjusted) for the non-statistically significant ferritin-MetS association in premenopausal women were smaller than for post-menopausal women and men, then lack of evidence for an association may not be due to low statistical power. It is not known to what extent adjustment for transaminases might have attenuated the significant association between ferritin and MetS described in this Croatian population as liver function tests were not measured in the study population.

Neither ferritin nor sTfR was associated with HbA1c, whether or not diabetes was included as a covariate in the models. This finding is consistent with the lack of association between ferritin and HbA1C reported in the 3876 participants of NHANES III (1988–1994) (180). Previously, Fernandez-Real et al. (181) and Rajpathak et al. (182) described significant weak correlations ($r = 0.14$ and $r = 0.12$) between sTfR and HbA1C in 221 men and 560 overweight individuals, respectively. However, in the first study, the correlation was unadjusted, and the second only adjusted for age and sex, and it is not known if metabolic and adiposity covariates might have attenuated the relationships. Of note, the associations between sTfR and HbA1C do not appear to have been evaluated using robust multivariate analyses in the existing literature. Therefore, further population-based studies are needed to confirm the absence of association between HbA1C and markers of iron metabolism after multivariate adjustment in general populations.

To the best of my knowledge, the present study is the first to investigate the association between sTfR and both MetS and IR by means of a robust multivariable analysis. I also extended the finding by Hamalainen et al. describing the absence of a significant independent relationship between STfR and MetS by performing additional adjustments for BMI, alcohol consumption, CVD, and diabetes. The key limitations of this study include of the relatively small number of pre-menopausal women and the inability to adjust for hepatic dysfunction since markers such as

transaminases were not measured in the original study. In the original project, there were no specific questions about the use of lipid-lowering medications, so associations with components of low HDL-C and high triglyceride could be underestimated since these components did not include individuals with prescribed medication, which might have affected these values. The cross-sectional design of the study means that it is not possible to provide evidence of a causal relationship between iron status and cardiometabolic risk factors. In addition, some findings may be due to chance.

The analysis in this chapter was conceived and conducted before the analyses and meta-analyses on ferritin and MetS described in Chapter 3. Supplementary analyses of the association between ferritin and MetS were performed so that they could be compared with the findings on STfR. The findings on the ferritin-MetS association in this Croatian population could have been part of the meta-analysis in Chapter 3. However, the work described in this chapter was in process of submission and peer-review for publication when Chapter 3 was being developed which meant that it was not eligible for inclusion in the meta-analysis. The results for the Croatian population, are consistent with the meta-analysis of the sub-group of studies without adjustment for liver injury markers.

In conclusion, this study showed that sTfR levels are associated with IR but not with MetS, independent of age, subclinical/chronic inflammation, smoking and alcohol habits, glycosylated haemoglobin, and CMD, in a population with a high prevalence of MetS and abdominal obesity. It is possible that sTfR level is a poor marker of erythropoiesis or iron metabolism in subjects with IR or hyperinsulinemia, and its levels are spuriously elevated and thus not associated with MetS or its components. There is a complex relationship between markers of iron status and cardiometabolic risk, with inconsistent associations with different markers. The next chapter examines the association between ferritin and different CMDs in a prospective study of a representative Scottish adult population.

Chapter 5

A prospective cohort study of the relationship between serum ferritin and incident cardiometabolic diseases in Scottish adults

5.1 Introduction

As mentioned in the introductory chapter, increased iron stores, reflected by high serum ferritin levels, have been associated with the development of T2D diabetes (115). Reports in the association between iron stores and other CMDs for which diabetes is also a risk factor, such as CHD, are inconsistent (183). Few studies have investigated the association between iron metabolism and CEVD. To date, no published study has focused on simultaneous evaluation of associations between iron stores and all the above CMDs in the same population or in nationally representative samples. In addition, threshold effects of ferritin concentration on the risk for CMD or the shape of the relationships have not been investigated in most of the previous studies.

In Scotland, despite the fact that the incidence of CHD decreased by around 30% between 2005 and 2014, CHD persists as a leading cause of illness and death (184). In the UK in 2014, Scotland had one of the highest prevalence rates of CVD (4.3%), along with the North of England (4.5%) (56). Prevalence of all types of diabetes has increased over the last decade in Scotland, from 3.2% to 5.1%, with decreasing mortality and stabilised incidence contributing to this pattern (185). Moreover, the prevalence of obesity, a well-known CMD risk factor, also increased between the 1990s and the 2000s (186), and unhealthy diet has been found to be more common in Scotland than in the rest of the UK (187). However, there is no information on iron biomarkers as a potential novel risk factor for CMD in the Scottish population.

The description of the relationship of serum ferritin with all types of CMDs in a population at high cardiovascular risk would allow a better understanding of the link between iron metabolism and overall cardiometabolic risk. In light of the above, I conducted a study to investigate the risk of diabetes, CHD, and CEVD in participants

in the 1995 and 1998 Scottish health surveys (SHeS), who were followed-up until 2011, providing a wide range of serum ferritin levels. These two surveys are the only ones (until recently) for which consent for data linkage was interpreted as including the diabetes register. The most recently available linked data provided information on all incident outcomes to the end of 2011.

5.2 Methods

5.2.1 Study population

The SHeS 1995 and 1998 included participants aged 16–74 years. The surveys randomly selected a nationally representative, general population sample (see The Scottish Health Surveys websites for more detail) (117, 118). All participants were interviewed for health and lifestyle behaviours, and consent for measurement of weight and height and collection of a blood sample was requested. The SHeS also has a prospective element on the basis of linkage to hospitalizations and mortality. This linkage is facilitated by the Information Service Division (ISD) which collects and updates data on deaths from the General Register Office and morbidity derived from hospital discharge data in Scottish Morbidity Records (SMR) and provides linkage to SHeS data(188). There is also a retrospective element with data - linked to morbidity since 1981. Around 90% of SHeS participants provided consent for data linkage (188). As a consequence of the wording of the consent for data linkage only data from the 1995 and 1998 SHeS have been linked to diabetes register data. There were 9,568 participants whose ferritin levels were measured and who agreed to have their data linked to the Scottish Morbidity Record (SMR), the Scottish Diabetes Register, and death records. Among these, I excluded cases of prevalent T2D, CHD, and CEVD at baseline (defined as interview day), subjects who had type 1 diabetes (T1D) at baseline or who developed T1D during the follow-up, cases with missing values for covariates (listed in data analysis section), and those below the set age limit (age ≥18 years). Appendix Figure 7 describes the selection of the analytical sample (*n*= 6497). The secondary analysis of the SHeS-linked dataset was approved by the Ethics Research Subgroup of the Centre for Population Health Sciences of the University of Edinburgh.

Biochemical and clinical variables

Methods for measurement of biochemical and clinical variables are the same as described in chapter 3 for the cross-sectional SHeS analysis. Physical activity levels, an additional variable used in this analysis, were estimated as a summary of self-reported work, walking, and sport activities in categories of intensity levels as inactive, light, moderate, and vigorous (189).

5.2.2 Cardiovascular outcomes and T2D

Incident CHD and CEVD were defined as any relevant code ICD-10(190) (I20-I25, I60-I67, G45) or ICD-9(191) (410-414, 430-437) for fatal and non-fatal episodes of these diseases recorded in hospital admissions during the follow-up period. Incident cases of T2D were taken from relevant codes ICD-10 and ICD-9 (E11-E14, 250) recorded during hospital admission during the study follow-up and from the linked data of the Scottish Diabetes Register which has been complete since 2004. Codes for unspecified diabetes were included as incident T2D assuming that most of these cases accounted for this type of diabetes.

5.2.3 Data analysis

Medians and their interquartile ranges and proportions were used for description of continuous and categorical study variables, respectively, in the whole sample and by sex-specific and self-reported menopausal status-specific quartiles of ferritin concentration. Trends of distribution of study variables across ferritin quartiles were tested by the Jonckheere-Terpstra test(192) (continuous variables) and χ^2 test (categorical variables). For the analyses of association, ferritin levels were used as both continuous and categorical variables. For the continuous approach, I calculated a sex/menopausal status-specific Z score for ferritin, after log-normalisation of the ferritin values. The Z score enabled reporting of the risk for CMD by increasing SD units of log-ferritin. The categorical approach involved the use of sex/menopausal-specific quartiles of ferritin, with the lowest quartile as the reference category. Cox regression models were used to examine the longitudinal associations between

ferritin and CMD. HRs were described as unadjusted, age- and sex/menopausal status-adjusted, and additionally adjusted for potential confounders, which were fibrinogen, GGT levels, smoking, alcohol consumption, total cholesterol, HDL-C, SBP and DBP, and BMI. This set of confounding factors was chosen on the basis of the possible influence of acute phase or subclinical inflammation, adiposity, liver dysfunction, and cardiovascular risk factors on values of ferritin and/or outcome variables. As mentioned in Chapter 3, the year of the survey was also part of the adjustment because there were changes in laboratories where measurements of total and HDL-C were taken in 1998, although the methods did not differ.

The proportional hazards assumption was tested by the Schoenfeld residuals test and graphical method. The follow-up years were calculated from the date of survey interview (1995 or 1998) to the first mention of T2D, cardiovascular event, death, or end of December 2011. Potential threshold effects of ferritin concentration were additionally investigated by comparing extreme quintiles and sextiles. Sensitivity analyses were performed on the basis of exclusion of subjects with clinically increased ferritin (>200 µg/L in women and >300 µg/L in men), potential liver disease (defined as GGT >84 IU/L in men and >44 IU/L in women), evidence of inflammation or infection (defined as fibrinogen levels >4.7 g/L in 1995 and >3.8 g/L in 1998), and sex/menopausal status-specific analyses.

Further adjustments for physical activity, self-reported hypertension, WC, and CRP levels as a more systemic inflammatory marker, were also conducted. The adjustment for CRP was only performed for the participants from the SHeS 1998 in which this marker was measured. Before conducting the association analyses, the continuous covariates with skewed distributions (fibrinogen, GGT, CRP, and WC) were log-transformed to approximate to normal distributions. To explore the shape of the relationship between serum ferritin and each incident CMD, I used restricted cubic splines with knots at the following percentiles of ferritin: 5^{th}, 27.5^{th}, 50^{th}, 72.5^{th}, and 95^{th}, as suggested by Harrell (193).

Survey weights were applied to adjust for disproportionate sampling, differing selection probabilities, and differential non-response, but unweighted analyses were also conducted to test the consistency of the effect estimates and confidence intervals

and are presented in the appendix material. The SHeS described the weighting method as follows: "*by applying two sets of weights: the first is needed in order to correct for the unequal probabilities of selection of addresses, and the second to correct for the unequal probabilities of selection of households and of individuals within households. These corrections are made by applying weights which are inversely proportional to the selection probabilities for the relevant postcode sectors and addresses. However, this weighting does not correct for variations in response level by region or by different groups of people. If there are such variations a further stage of weighting can be applied. For the Health Survey data it was decided to apply two further weights: the first to correct for differing rates of response between the seven regions; and the second to correct for the under-representation of men and young people in the achieved sample, so that the age and sex distribution of the sample would resemble that for the Scottish population*". All analyses were processed using STATA 14.0 software (Statistics/Data Analysis, Stata Corporation, 4905 Lakeway Drive, College Station, TX 77845, USA, 800-STATA-PC).

5.3 Results

Differences between included and excluded individuals in this analysis are shown in Appendix Table 30. The excluded subjects had higher values of all cardiovascular risk factors. These differences were expected taking into account the fact that among the excluded individuals, some had T2D and CVD. Serum ferritin and GGT levels were not significantly different between included and excluded individuals.

The study variables by sex/menopausal status-specific ferritin quartiles are described in Table 15. Age, fibrinogen, GGT, BMI, total cholesterol, and blood pressure significantly increased across ferritin quartiles. The same pattern was observed for prevalence of current smokers and higher alcohol intake, and for the proportion of subjects who developed diabetes, CHD, and CEVD during follow-up. In contrast and as expected from the other risk factor patterns, HDL-C levels decreased with higher levels of ferritin. There was also a trend of a slightly increasing higher proportion of participants from SHeS 1995 vs. participants from 1998 throughout ferritin quartiles (Table 15). Ferritin levels were higher in men than women, and higher in post-menopausal women than pre-menopausal women ($P < 0.05$, data not shown).

During follow-up, 4.9% of the participants developed T2D, 5.3% developed CHD, and 2.3% developed CEVD. The incidence of T2D and CEVD was higher in post-menopausal women than in men [T2D 8.1% vs. 5.5%, CEVD 4.7% vs. 2.2%] and the incidence of CHD was comparable in these 2 groups, i.e. 7.0% and 7.2%, respectively. In pre-menopausal women, the incidence of CHD, T2D, and CEVD was 2.4%, 1.7%, and 1.3%, respectively. Deaths from CHD and CEVD occurred in 1.0% and 0.4% of the participants, respectively, while T2D was reported as the cause of death for only 2 people. Proportions of deaths from CHD were comparable in post-menopausal women and men at 1.4% and 1.3%, respectively. The proportion of deaths from CEVD was higher in post-menopausal women than men, i.e. 0.9% vs. 0.3% and was 0.2% in post-menopausal women.

Table 16 shows the HRs for the longitudinal association of serum ferritin levels, as Z score and quartiles, with the different CMDs. In unadjusted and age- and sex/menopausal status-adjusted models, ferritin as a continuous variable and ferritin levels in the highest quartile (compared to the lowest quartile) were positively and significantly associated with all types of incident CMD. In fully adjusted models, all the HRs for associations by quartiles were in general attenuated as compared to unadjusted models. The fully adjusted hazard of T2D was 71% higher in individuals with high levels (highest quartile) of ferritin compared to people with low concentrations (lowest quartile), and an increase in SD units of log ferritin was associated with a 22% increase in hazard ($P \leq 0.001$). The associations of ferritin with CEVD and CHD were no longer statistically significant after full adjustment for covariates although effect estimates remained above 1 and there was the suggestion of a dose-response relationship (Table 16).

Table 15 Baseline characteristics of participants from Scottish Health Surveys 1995-1998 with relevant data available and proportions developing outcome by sex- and menopausal status-specific quartiles of ferritin level in the study cohort (n=6497) [weighted values]*

		Ferritin (µg/L)				
	All	**Q1**	**Q2**	**Q3**	**Q4**	**P for trend**
Ferritin lrange by sex/menopausal status (µg/L)*						
Premenopausal women (Pre-W) (*n=2239*)	2.0-950	2.0-18	19-30	31-47	48-950	
Postmenopausal women (Post-W) (*n=1343*)	3.0-1000	3.0-34	35-58	58-91	92-1000	
Men (*n=2915*)	3.0-2251	3.0-61	62-96	97-151	152-2251	
Variables						
Age	41(31-52)	39(29-50)	40(30-50)	40(31-52)	44(34-54)	<0.001
Pre-W/Post-W/ Men (%)	32.9/16.6/50.5	32.9/16.4/50.7	32.2/16.9/50.9	33.3/17.2/49.5	33.0/15.9/51.1	0.887
Ferritin (µg/L)	61(32-108)	25(13-43)	62(28-78)	91(42-116)	161(86-216.8)	<0.001
Fibrinogen (g/L)	2.9(2.4-3.5)	2.8(2.3-3.4)	2.9(2.4-3.4)	3.0(2.5-3.6)	3.1(2.6-3.7)	<0.001
GGT (IU/L)	20(14-32)	17(13-25)	18(14-27)	21(15-33)	27(18-45)	<0.001
Smoking status (%)						
Never smoker	39	45.4	41.1	36.1	33.0	<0.001
Ex-regular or Ex-occasional smoker	26.3	24.4	26.3	26.1	28.4	
Current smoker	34.7	30.2	32.6	37.8	38.6	
Alcohol consumption. Categories of rating units/week Prevalence (%)						<0.001
Never drank	4.2	5.1	5.4	3.6	2.6	
Ex-drinker	2.8	3.2	3.1	2.7	2.4	
Trivial drinker/Non-zero but under 1	10.3	12.6	10.7	9.1	8.5	
1-20	63.4	64.5	62.9	65.4	60.6	
≥ 21	19.3	14.6	17.8	19.2	26.0	
BMI (Kg/mts^2)	25.7(23.1-28.6)	24.9(22.7-27.8)	25.3(22.8-28.1)	25.8(23.3-28.5)	26.6(24.1-29.9)	<0.001
HDL-cholesterol (mmol/L)	1.4(1.2-1.7)	1.4(1.2-1.7)	1.4(1.2-1.7)	1.4(1.2-1.7)	1.4(1.1-1.7)	0.007**
Total cholesterol (mmol/L)	5.4(4.7-6.2)	5.3(4.6-6.0)	5.4(4.6-6.2)	5.4(4.8-6.2)	5.6(4.9-6.4)	<0.001

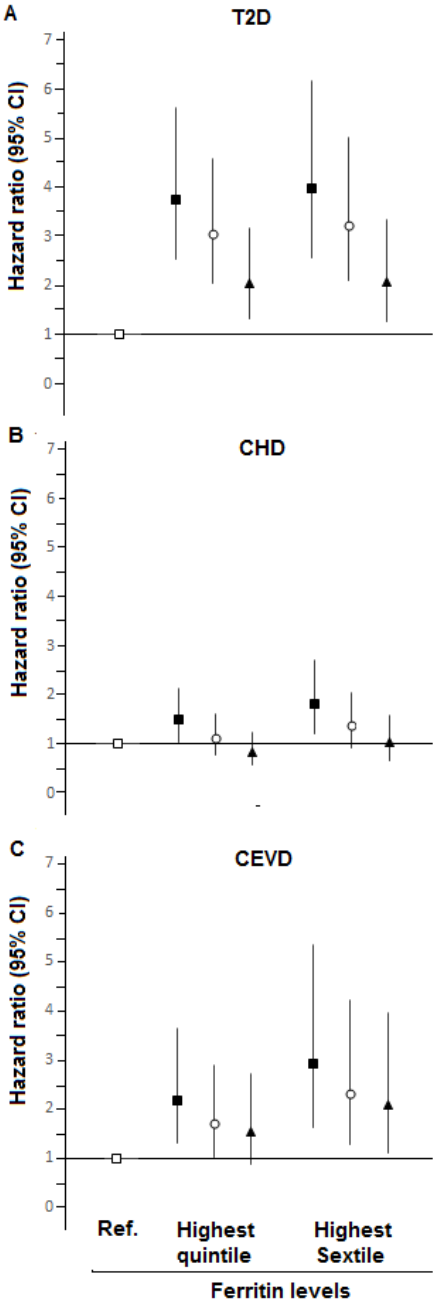
	Ferritin (µg/L)					
	All	**Q1**	**Q2**	**Q3**	**Q4**	**P for trend**
Systolic blood pressure (mmHg)	126(117-137)	125(117-135)	125(116.5-135)	125.5(116.5-137)	128.5(118-140)	<0.001
Diastolic blood pressure (mmHg)	70(63-79)	69(62-77)	69(62-78)	70(64-78)	73(65-81)	<0.001
Incident diabetes (%)	4.9	3.3	3.4	4.5	8.7	<0.001
Incident coronary *heart disease* (%)	5.3	4.5	4.6	4.8	7.6	<0.001
Incident cerebrovascular disease (%)	2.4	1.8	2.0	2.3	3.4	0.003
Survey 1995/1998 (%)	50.1/49.9	44.6/55.4	50.8/49.2	52.6/47.4	52.6/47.4	<0.001
***Samples sizes, quartiles and ranges of ferritin levels are based on unweighted values. ** Negative trend across quartiles is more evident in terms of rank values.**						

Table 16 HRs and 95% CI for the incidence of diabetes and cardiovascular diseases by serum ferritin levels (weighted analysis)

	Type 2 diabetes			Coronary heart disease			Cerebrovascular disease		
	Unadjusted	Age- and sex/menopausal status- adjusted	Fully adjusted*	Unadjusted	Age- and sex/menopausal status- adjusted	Fully adjusted*	Unadjusted	Age- and sex/menopausal status-adjusted	Fully adjusted*
Z score of log-ferritin	**1.57 (1.39-1.78) P<0.001**	**1.45 (1.28-1.64) P<0.001**	**1.22 (1.07-1.39) P=0.002**	**1.22 (1.08-1.38) P=0.001**	**1.11 (1.00-1.25) P=0.049**	1.02 (0.90-1.15) P=0.669	**1.28 (1.09-1.51) P=0.002**	**1.18 (1.01-1.37) P=0.029**	1.12 (0.95-1.33) P=0.163
Ferritin									
Quartile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Quartile 2	1.01 (0.67-1.50)	0.95 (0.64-1.41)	0.99 (0.66-1.50)	1.02 (0.71-1.46)	0.95 (0.66-1.36)	0.89 (0.61-1.29)	1.10 (0.66-1.82)	1.04 (0.62-1.73)	1.05 (0.63-1.75)
Quartile 3	1.34 (0.92-1.97)	1.23 (0.84-1.80)	1.10 (0.73-1.65)	1.04 (0.74-1.47)	0.93 (0.66-1.31)	0.79 (0.56-1.13)	1.23 (0.75-2.00)	1.11 (0.68-1.81)	1.05 (0.63-1.75)
Quartile 4	**2.73 (1.94-3.85)**	**2.28 (1.61-3.21)**	**1.59 (1.10-2.34)**	**1.70 (1.23-2.36)**	1.35 (0.97-1.87)	1.07 (0.76-1.51)	**1.86 (1.18-2.95)**	1.52 (0.96-2.40)	1.36 (0.81-2.27)
P for trend	**P<0.001**	**P<0.001**	**P=0.006**	**P=0.002**	P=0.070	P=0.700	**P=0.007**	P=0.065	P=0.253

*** Adjusted for age, sex/menopausal status, fibrinogen levels, GGT levels, alcohol intake, smoking, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, body mass index and year of survey.**

Figure 7 Comparison of hazard ratios for type 2 diabetes (T2D), coronary heart disease (CHD) and cerebrovascular disease (CEVD) for quintiles and sextiles of ferritin levels (v. respective lowest categories) with different levels of adjustment



Ferritin highest quintile: Premenopausal women (Pre-MW) 53-950 µg/L, Postmenopausal women (Post-M) 102-1000 µg/L, Men 169-2251 µg/L. Ferritin highest sextile: Pre-MW 58-950 µg/L, Post-MW 114-1000 µg/L, Men 183-2251 µg/L. □ Reference (lowest quintile, sextile). ■ Unadjusted. ○ Adjusted for age and sex/menopausal status. ▲ Adjusted for age, sex/menopausal status, fibrinogen levels, GGT levels, alcohol intake, smoking, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, body mass index and year of survey. The above analysis included survey weights.

When I explored the effect of using quintiles or sextiles to determine if the highest category of ferritin strengthened the associations with CMD, there was a suggestion of an effect for CEVD, although confidence intervals for HRs overlapped (Figure 7C). The hazard of CEVD was approximately double in individuals with ferritin in the highest sextile compared to those in the lowest sextile [fully adjusted (HR) 95% CI 2.08 (1.09–3.94), P = 0.024]. The association with T2D was slightly stronger when comparing extreme quintiles or sextiles than when comparing extreme quartiles (Figure 7A), and no significant associations with CHD were observed (Figure 7B). It is worth noting that the sex/menopausal status-specific cut-off points for the highest quintile and sextile were in the clinical normal range for serum ferritin (≤300 μg/L) (Figure 7).

In the sensitivity analyses, the exclusion of individuals with serum ferritin >300 μg/L or further adjustment for self-reported hypertension or physical activity and WC did not substantially affect the associations previously described. The ferritin-CEVD association remained unaffected when cases of subarachnoid haemorrhage were excluded (n=16). In participants from SHeS 1998, further adjustment for CRP levels did not alter the significant association between serum ferritin in the highest quartile (vs. lowest quartile) and incident T2D [unadjusted HR (95% CI): 2.18(1.22–3.90), P = 0.008; fully adjusted HR (95% CI): 2.16(1.20–3.89), P = 0.010]. In this same sub-cohort, ferritin and incident CHD and CEVD were not significantly associated, and additional adjustment for CRP did not modify these non-significant associations.

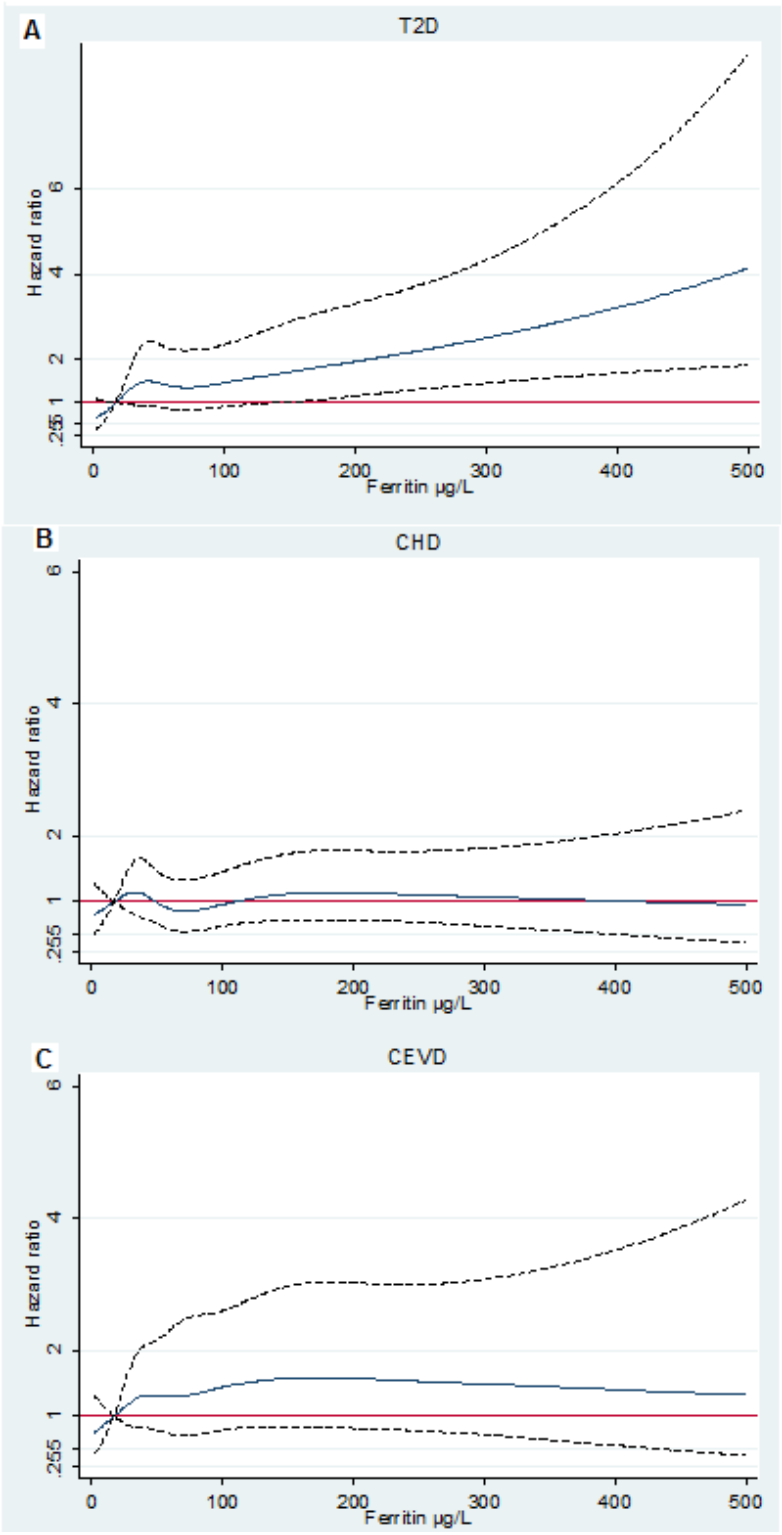
When the analysis was restricted to individuals without signs of inflammation and/or hepatic injury (n=5626), the association between ferritin, as upper quartile or continuous variable, and T2D was attenuated [ferritin Z score HR (95% CI) 1.12 (0.97–1.31), P = 0.105; higher quartile vs. lowest HR (95% CI) 1.32 (0.87–2.09), P = 0.177]. However, by comparing extreme quintiles, the association was still borderline statistically significant [HR (95% CI) 1.67 (1.02–2.75), P = 0.041]. The association between ferritin (extreme sextiles compared) with CEVD remained similar [HR (95% CI) 2.03 (1.01–4.08) P = 0.045].

I also evaluated the association with ischaemic CEVD specifically (92 incident cases) but no significant association was found with ferritin as a continuous variable or quantile (quartiles, quintiles, sextiles).

Unweighted results of the description of study variables and Cox regression analyses showed similar patterns described above, although with some differences in P values for some associations (Appendix Tables 31-32 and Appendix Figure 8).

Although there was no evidence of interaction between sex or menopausal status and ferritin levels in respect to developing CMD ($P > 0.05$ in weighted and unweighted interaction tests), I conducted analyses stratified by sex and menopausal status, with and without exclusion of individuals with particular characteristics as described for the sensitivity analyses in the whole sample. The purpose of these extra analyses was to compare the findings with a recent large European, multi-centre study on iron markers and incident T2D. In these analyses stratified by sex and menopausal status, the statistically significant ferritin Z score-T2D association persisted only in men [HR (95% CI) 1.20 (1.01–1.43), P = 0.033; pre-menopausal 1.25 (0.92–1.69), P = 0.149; post- menopausal women 1.14 (0.87–1.49), P = 0.325]. When cases with ferritin levels above the normal range were excluded, a different pattern of association between ferritin Z score and T2D was observed in pre- menopausal women [HR (95% CI) 1.32 (0.96–1.82), P = 0.079] and men [HR (95% CI) 1.17 (0.93–1.46) P = 0.159]. A similar pattern was observed when the analysis was restricted to subjects without high levels of fibrinogen and/or GGT [HRs (95% CI) pre- menopausal women 1.38 (0.95–1.99), P = 0.085; men 1.11 (0.92–1.34), P = 0.246); post- menopausal women 0.92 (0.66–1.29), P = 0.658]. There were no statistically significant associations between serum ferritin, as Z score or quantiles, and CEVD or CHD in the analyses by sex and menopausal status. Combining data for pre- and post- menopausal women in a single category and adjusting for menopausal status, did not result in significant associations across the above mentioned sensitivity analyses.

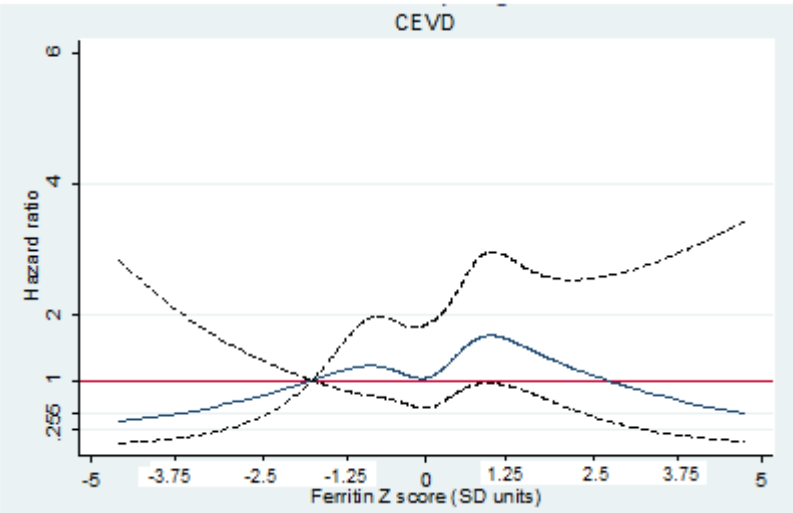
Figure 8 Risk of cardiometabolic diseases by serum ferritin levels.



Adjusted hazard ratios for A) T2D, B) CHD, and C) CEVD by serum ferritin levels .Ferritin levels higher than 500 µg/L were not used because confidence intervals tended to be very wide in this graphical analysis.

The relationship between serum ferritin levels and incident T2D was approximately linear (Figure 8A). In contrast with the findings by using sextiles of ferritin, the association between ferritin and CEVD was not observed when using cubic splines in terms of ferritin levels (Figure 8B). This is perhaps because the ferritin values used for the evaluation of non-linear relationships were not sex/menopausal status-specific as they were in the analyses with ferritin as sextiles. When sex/menopausal status-specific Z scores of ferritin were used in the cubic splines analysis, a threshold effect of higher risk of CEVD appeared around +1.20 SD of log-ferritin, although confidence intervals in that region of the graph still included 1 (Figure 9). The above findings on the shape of the association between ferritin or ferritin Z score and incident CEVD remained unmodified by using six knots instead of five in the cubic spline analysis.

Figure 9 Adjusted hazard ratios for CEVD by sex/menopausal specific Z score of ferritin levels.



5.4 Discussion

In the present study, I have reported the associations between iron stores and incidence of several CMDs in a nationally representative population at high cardiovascular risk over a mean follow-up of 14 years. I found a statistically significant association between high ferritin and development of CEVD when I used

higher percentiles to define high ferritin concentration. Interestingly, these cut-off points were still within the normal reference values of ferritin. The findings also confirm previous observations of an association between serum ferritin and T2D and no evidence of an association with CHD. The association between ferritin levels and T2D seemed to be more consistent in pre-menopausal women than in men in sensitivity analyses.

5.4.1 Ferritin and T2D

The results for the association between serum ferritin and incident T2D are consistent with the positive and significant association reported in a meta-analysis of prospective studies published up to 2012 (1). In this meta-analysis, the pooled relative risk (95% CI) for incident T2D for the highest ferritin quintile vs. the lowest quintile was 1.73 (1.35–2.22) (115). The association in the meta-analysis is slightly stronger than that in the Scottish population studied here [HR (95% CI) 1.59(1.10–2.34), P = 0.006], presumably because several studies of the meta-analysis lacked adjustment for transaminase levels. In the present study of Scottish population, the HR for the association between highest compared to lowest quartile of ferritin levels and incident T2D decreased by 16% when GGT levels were added to the adjustment model [from 1.91 (1.32–2.77), P = 0.001 to 1.60 (1.10–2.34), P = 0.014]. Some few studies failed to find a significant independent association between serum ferritin and development of T2D. A case-cohort study of the Atherosclerosis Risk in Communities study (ARIC) (599 cases and 690 controls) did not find a significant association between ferritin levels in the highest quintile (v. the lowest) and T2D after adjustment for BMI and traditional cardiovascular risk factors [HR 95% CI 0.81 (0.49–1.34) (20). However, the study did not disclose which covariate/s had more weight in the attenuation of the association. In a recent age and sex-matched case-control prospective study (327 cases, 641 controls) involving Japanese workers, the association between ferritin and incident T2D was weakened after further adjustment for transaminase and lipid levels [HR (95% CI) 1.40 (1.01–1.93), P = 0.02 before adjustment; HR (95% CI) 1.20 (0.86–1.67), P = 0.16 after adjustment] (194). It is posssible that limited statistical power explains the non-statistically significant findings in the ARIC and Japanese cohorts.Recently, a large study conducted in a sub-cohort of the European multi-centre InterAct study was published, which

examined the association of serum ferritin and three other serum iron biomarkers (transferrin, serum iron, and percentage of transferrin saturation (TSAT) with incident T2D (195). This is the most detailed study to date on iron metabolism and T2D. Given the large sample size, the authors of this study were able to conduct sex-specific analyses with multiple sensitivity analyses to characterise the associations. The authors found a significant association between serum ferritin and T2D in both sexes. However, the association was markedly attenuated in men but not in women when individuals with signs of inflammatory or hepatic disease, high alcohol intake, and who were overweight were excluded from the analysis. Similarly, the association was strengthened for women but not for men when their analysis was restricted to ferritin values lower than 1000 μg/L. The findings from the Scottish population are partly consistent with the patterns from the InterAct study. In the prospective Scottish study population, the significant association between ferritin and T2D persisted for men but not for women after adjustments. However, as described in the results section, by limiting the analysis to individuals without any sign of inflammatory or hepatic disease and with ferritin levels within the normal range, there was a higher effect estimate for association in pre-menopausal women than in men with borderline statistical significance.

The above findings of a more consistent association between iron status and CMD among premenopausal women than among men within a healthier sub-population, do not appear to be explained by exclusion of cases of T2D and/or high ferritin in men. The proportion of cases with high fibrinogen and/or GGT levels in the highest ferritin quartiles in men and women were comparable (18% in premenopausal women and 21.3% in men). Moreover, a higher relative proportion of T2D cases in the highest ferritin quartile were excluded among pre-menopausal women (21% based on reduction of absolute proportions developing diabetes during follow-up from 3.8% to 2.6%) than among men (13%- absolute proportions before and after exclusions 10.3% and 8.9%) when individuals with potential clinical inflammation or liver disease were removed. The difference by sex in sensitivity analyses may be partly related to statistical artefact. Relative risks (or similar effect estimates such as hazard ratios) for detrimental factors tend to be higher in sub-populations with a lower absolute risk of the outcome of interest, such as women in this case, in

comparison with sub-populations with a higher absolute risk, such as men. In this analysis, absolute proportions of people developing T2D in the highest quartile of ferritin during follow-up for premenopausal women and men were 3.8% and 10.3%, respectively. In the lowest quartile ferritin, the absolute risk was 0.9% in premenopausal women and 3.7% in men. Higher relative risks may therefore be expected in the group at lower absolute risk, ie premenopausal women. In addition, during the long follow-up period of this study, in comparison with men, changes in iron stores and cardiometabolic risk may have been more marked in premenopausal women during subsequent perimenopause and postmenopause. However, further research and large longitudinal studies with repeated measurements of ferritin are required to test this hypothesis.

Despite the similarities in patterns, it is important to bear in mind that sex-specific sensitivity analyses conducted in both the InterAct study and the SHeS could lead to chance findings due to multiple testing. Therefore, any sex-specific difference should be viewed with caution until further studies confirm this pattern.

Another important aspect of the current evidence on the association between ferritin and T2D is whether there is potential for residual confounding. Two analyses in sub-cohorts of the EPIC-Norfolk and the EPIC-Potsdam study included a broad set of covariates in their multi-variable models including classic risk factors and also adiponectin, interleukin 6, and fetuin, but none of these covariates attenuated the association of ferritin with T2D significantly (26, 164).

In the present work, the association between ferritin and T2D was linear with no appreciable threshold effects, and this is consistent with the pattern reported in the EPIC-InterAct study. Potential threshold effects have been previously suggested in other studies, but those observations could be influenced by low statistical power (26, 115).

5.4.2 Ferritin and CEVD

As previously mentioned, there have been very few studies on ferritin and CEVD in general populations, and those that exist show inconclusive findings. Two longitudinal studies involving 1,134 Dutch post-menopausal women (aged 40–70

years, mean follow-up, 4.3 years) (196) and a sub-cohort (n = 1612) of the Busselton Health study (age 40–89 years,17-years of follow-up) (197) reported similar positive associations between serum ferritin levels in the highest tertile (vs. lowest) and stroke of any subtype but without reaching statistical significance in fully adjusted models [HR(95%CI) 1.45(0.76–3.85) and 1.43(0.78–2.64), respectively]. Analyses by sex in the Busselton Health study did not show statistically significant associations either. Both studies had comarable age ranges and number of incident cases of stroke (Dutch cohort, 63 cases; Busselton Health study's sub-cohort, 55 women and 63 men). In the Dutch cohort of post-menopausal women, by using serum ferritin ≥200 μg/L compared to ferritin lower than that cut-off point, the positive association between ferritin and stroke of any subtype was borderline statistically significant [HR(95%CI) 1.77(1.03–3.05)]. Although the cut-off point for highest tertile of ferritin for men in the Busselton Health study was 233 μg/L, for women the cut-off point for this tertile was only 122 μg/L. Therefore, it is uncertain whether by comparing extreme values of ferritin, the association with stroke might have been strengthened in the Busselton Health Study.

In this chapter of my thesis, by exploring additional higher cut-off points for increased ferritin, the association between serum ferritin and CEVD became more evident. Since CEVD is less common than T2D and CHD, it is likely that clearer associations can be observed when incident and/or prevalent cases are more concentrated into very high categories of distribution of a predictor variable, such as iron stores. Despite using sextiles of ferritin distribution, the cut-off points defining increased ferritin were still within the normal range for ferritin levels, and the exclusion of subjects with ferritin values > 300 μg/L did not affect the ferritin-CEVD association. This suggests that there may be an increased risk of CEVD in the general population that does not have extremely high concentrations of stored iron if this is not a chance finding. However, a graphical evaluation of the ferritin-CEVD relationship by using standardised values of ferritin in my analysis suggested, a threshold effect. Studies with more incident CEVD cases are required to establish whether this is a true finding, since statistical tools for testing non-linearity demand high statistical power. My analysis, to the best of my knowledge, is the first attempt

to graphically describe the shape of the association between serum ferritin level and the risk of CEVD.

In the same Dutch cohort of post-menopausal women mentioned previously, the positive association between ferritin in the highest tertile and stroke was strengthened when only cases of ischemic stroke [HR (95%CI) 2.23 (1.05–4.73)] were considered (196). In contrast, in the present study involving the Scottish population, increased ferritin was not statistically significantly associated with ischaemic CEVD and this could be due to loss of power.

The association between ferritin and CEVD has also been investigated by describing the association between ferritin and sub-clinical carotid atherosclerosis, a risk factor for stroke. As with stroke, the findings are conflicting, but low statistical power may contribute to the apparent lack of association. In the 1990s, cross-sectional and prospective analyses in the Brucneck study showed an association between ferritin levels and sonographically assessed carotid atherosclerosis in 847 men and 826 women aged 40–79 years (198). In contrast, a study involving 105 and 66 Finnish men aged 50–60 years with and without cardiovascular disease, respectively, did not find serum ferritin, transferrin, or iron intake associated with carotid bifurcation atherosclerosis (199). The ARIC study did not find a significant association between ferritin and asymptomatic carotid atherosclerosis when 169 cases and 152 controls, matched for sex, were compared [adjusted OR (95% CI) 1.0 (0.851.19)] (200). Recently, a study involving 1,178 post-menopausal women, which used a high-resolution ultrasonography to assess carotid atherosclerosis, reported a significant association [OR for increased bilateral carotid intima-media thickness (95%CI) 1.58 (1.05–2.31), $P < 0.05$ for the top compared to bottom quartile of ferritin] (201).

The potential mechanisms underlying the association between iron excess and stroke are still unclear, but endothelial alterations might play a key role in the aetiology of both ischemic and haemorrhagic subtypes. In terms of the ischaemic subtype, atherosclerosis would be a possible mediating process. As a consequence of its pro-oxidant and deleterious effects on endothelial cells, iron may favour thickening of the intima-media in the main blood vessels to the brain and contribute to consequent

occlusion of the vascular lumen (202). In the case of haemorrhagic stroke, the literature has focused on iron as a cause of oxidative brain injury after intracerebral haemorrhage rather than as a potential risk factor (203). On the other hand, in my view, since long-standing hypertension is the most common trigger for haemorrhagic stroke (204), iron-related mechanisms for hypertension could support a link between iron stores and stroke. In fact, higher serum ferritin levels have been found in individuals with essential hypertension than in subjects without hypertension (205). Endothelial dysfunction, defined as the loss of vasodilation capability in blood vessels, which is strongly associated with hypertension, has been shown to improve with iron chelation therapy in a general population (206). Iron seems to directly inactivate nitric oxide (NO) synthase in the endothelium, an enzyme which catalyses the releasing of NO to promote vasodilation of the blood vessels (206). The negative impact of iron on this enzyme also appears to be via oxidative effects that would involve membrane lipid and low-density lipoprotein peroxidation (206). However, no recent experimental studies further investigated these mechanisms. Despite the above evidence, in multivariate models, blood pressure and/or self-reported hypertension did not substantially affect the ferritin-CEVD association reported here.

5.4.3 Ferritin and the lack of an independent association with CHD

Serum ferritin levels were associated with CHD in unadjusted and age/sex-menopausal status-adjusted models but not in the fully-adjusted model used in these analyses. In this latter model, several cardiovascular risk factors, such as cholesterol markers, SBP, and BMI, markedly attenuated the ferritin-CHD association (data not shown). The lack of an independent association is consistent with the findings from a recent meta-analysis by Das et al. on several iron markers and CHD which evaluated 17 prospective studies with a total of 9,236 cases of CHD and 156,427 participants (183). In this meta-analysis, the pooled association between serum ferritin in the highest tertile (vs. lowest tertile) and CHD was not significant [OR 95% (CI) 1.03 (0.87–1.23)]. Paradoxically, transferrin saturation was significantly and inversely associated with incident CHD. The authors acknowledged difficulties in inferring causality due to potential reverse causality and residual confounding. However, they did not ignore the likely role of anaemia in the inverse association, since a higher

iron status could prevent the onset of anaemia, which is associated with symptomatic CHD(183). It is still unclear why the directions of associations with iron status markers are inconsistent. If iron deficiency and anaemia are related to CHD, the effect estimates for the relationship with serum ferritin should show an inverse significant pattern as well. This discrepancy reinforces the notion that the iron markers are differentially associated with cardiometabolic risk through mechanisms other than iron metabolism.

Interestingly, as will be seen and discussed in the sixth chapter of this doctoral research thesis, serum ferritin showed an inverse association pattern with CHD in people with T2D, but underlying mechanisms for this disease-specific pattern have yet to be elucidated. It is unclear whether the presence of people with T2D within general populations may distort a positive association between ferritin and CHD in people without diabetes. Several studies from the meta-analysis of Das et al. included diabetes in their sets of covariates and, apparently, no modification of the ferritin-CHD association by diabetes status was observed. However, if the ferritin-CHD pattern in T2D appears to be different from the expected positive pattern in general populations, future studies in large cohorts, where there could be many more cases of diabetes, should include sensitivity analyses after excluding people with diabetes. In the present study, the Scottish cohort was diabetes-free at baseline.

The fully adjusted effect estimate described in this chapter for the non-significant positive association between high ferritin (comparison of extreme quartiles), [HR (95%CI) 1.08 (0.76–1.52)] is similar to those reported in three studies out of ten from the meta-analysis by Das et al. on ferritin and CHD (183). Among the remaining seven studies, four reported effect estimates much lower than 1.0, and three, higher than 1.0. However, Das et al. did not find any source of statistically significant heterogeneity among factors of location, degree of adjustment for confounders, sex, or case definition across meta-regression analyses (183).

5.4.4 Limitations and strengths

Several limitations need to be acknowledged. SHeS did not measure serum triglycerides, and it is unknown to what extent this risk factor could have attenuated the associations found. Lipid- and blood pressure-lowering treatments were not part of the adjustments owing to many missing values for medications within the sample selected. The analysis also lacked evaluations for other iron markers. Two issues may have led to underestimating the total number of cases of T2D and people with undiagnosed diabetes may have been included in the non-diabetic population. First, there could be an underestimation of incident T2D cases between 1995 and 2003, because cases of T2D for people who died before 2004 were only identified using information on hospital admissions, since the Scottish Diabetes Register is only thought to have been complete since 2004. There is also potential for erroneous inclusion of people with undiagnosed diabetes both at baseline and follow-up in the non-diabetic group. However, the above issues did not affect the statistical power to find a consistent association between ferritin and T2D in the Scottish population, and this may be related to the long follow-up of this study, which is the longest to date.

There was a high proportion of excluded cases with missing values for exposure/outcome variables or covariates (62.3%) and this may have introduced bias in the associations found. Estimation of outcome variables might have been affected by the loss of follow-up due to no link to the SMR. If the outcomes were overestimated, the associations with serum ferritin may have been biased toward the null hypothesis, as in the case of CHD. If the outcomes had been underestimated, it is possible that the loss of statistical power led to weaker associations, as observed for CEVD. Values of ferritin and biochemical covariates were conditioned to blood sample and performance of laboratory measurement. Eligibility for a blood sample in the SHeS required the participant not to be pregnant, and not to have bleeding disorders or be under anticoagulant treatment. Although in general, the response rate.for blood samples was around 78-80%, the measurements of the analytes in the samples were highly variable and thus not complete for all the subjects. The causes of failure to determine the different analytes in the blood sample were not described in the SHeS, but usually, this is related to haemolysis of the sample, which is the rupture of red blood cells. Haemolysis interferes with chemical assays or signal of

detection of ferritin and other analytes in laboratory spectrometric systems(207). However, not all biochemical assays are affected by haemolysis and this explains the inconsistency of having incomplete data (e.g. some cases with transaminase measurement but not ferritin).

Multiple imputation data techniques could potentially help to counteract the negative effect of missing values by replacing missing values with hypothetical values modelled on the basis of explanatory variables of the missingness (208). On the other hand, in the present SHeS analysis imputation models may be misleading since the causes of the missingness for each particular variable are not entirely clear, or if clear, these were not recorded and assumptions made during imputation may introduce different forms of bias. Another potential disadvantage of an imputation approach in this chapter is that methods for imputing missing values for categorical variables or with a skewed distribution such as ferritin, are still a developing area(208). Although imputation for the analyses with continuous values of the log-normalised ferritin Z score is feasible, imputation for the analyses with ferritin as quintiles would be more problematic, since quintiles are calculated from ferritin in its original units. In terms of plausibility in comparison to findings of other studies, the findings in this chapter do not appear to be seriously biased, but future studies on ferritin and cardimetabolic risk could compare results of analyses using complete case analyses and imputed data approaches because to date, there are not studies covering this methodological limitation.

Time trends in background interventions for prevention and treatment of cardiovascular disease may mean that it is inappropriate to extrapolate findings from the SHeS analysis to contemporary populations given the long follow-up period for SHeS participants. For instance, the proportions of people who take aspirin and statins have increased over that time period and these treatments have been linked (along with angiotensin converting enzyme inhibitors, thrombolysis, and coronary artery bypass graft surgery) to a 25-55% of the fall in cardiovascular mortality rates in Scotland (2000-2010)(209). It is not clear how this might have affected the association between ferritin and cardiovascular outcomes.

To the best of my knowledge, this study is the first to have simultaneously evaluated the association between ferritin and incidence of several CMDs. Moreover, this is the first study in a nationally representative population. The study also explored different upper categories of ferritin concentration in relation to the risk of CMD and evaluated the potential non-linear relationships between ferritin and each CMD outcome.

In conclusion, serum ferritin levels were positively and independently associated with incident T2D and with incident CEVD if higher cut-off points for upper ferritin levels were considered. The lack of an independent association between serum ferritin and CHD reported in previous studies was confirmed in this Scottish population. Further studies on ferritin and CEVD are required to confirm the association described here. The next chapter investigates the association between ferritin and CVD in people with T2D.

Chapter 6

Cross-sectional and longitudinal analyses of serum ferritin and cardiovascular disease in people with type 2 diabetes

6.1 Introduction

Little attention has been paid to the possible involvement of iron in the development of chronic complications of diabetes. In the general population, iron has been hypothesised to promote the development of the atherosclerotic plaque through increased oxidative stress as a consequence of the pro-oxidant properties of iron (11).

Iron imbalance could potentiate the risk for cardiovascular outcomes in people who already have T2D. So far, only one cross-sectional study involving 424 people has tested this hypothesis. The authors reported an apparently paradoxical decreased prevalence of macroangiopathy in men with T2D and increased serum ferritin levels, but adjustment for covariates was not performed (210). I did not find any previous longitudinal or larger cross-sectional studies evaluating the possible association between serum ferritin levels and cardiovascular events in people with T2D.

In light of the above, I investigated potential associations of serum ferritin with CHD and CEVD with adjustment for covariates in two different cohorts of people with T2D, one from Scotland and the other from Catalonia, Spain using both cross-sectional and prospective study designs. This multi-cohort approach was used to determine whether findings were consistent despite cardiovascular risk differences between British and Mediterranean populations.

6.2 Methods

6.2.1 Study population

ET2DS

The Edinburgh Type 2 Diabetes Study (ET2DS) has been described in full previously (211). The ET2DS is an on-going prospective study with data available for eight years of follow-up in this study. Patients with T2D aged 60–75 years were selected at random from the Lothian Diabetes Register, a comprehensive register of patients with diabetes living in Lothian, Scotland, UK, which was established in 2001. Baseline attendees (n = 1,066) have previously been shown to be representative of all those randomly selected to participate (n = 5,454) and therefore representative of the target population of older people with T2D in the general population [14]. At year 1, 939 participants visited a liver assessment clinic, and ferritin levels were measured for 876 of those participants. Therefore, year 1 was used as baseline for this study and the mean follow-up duration for the present analysis was 6.1 ± 1.7 years. Of the 876 subjects with ferritin measurements, I excluded 55 individuals with missing values for covariates, resulting in a final sample of 821 participants.

SIDIAP study – Primary Care Centres in Catalonia

This cross-sectional study is based on the SIDIAP system, a computerised database containing anonymised patient records for the 5.8 million people registered with a GP in the Catalan Health Institute (the regional health authority). The SIDIAP database includes data from a software called ECAP used by general practitioners to record clinical information on patients (demographic characteristics, consultations with GPs, diagnoses, clinical variables, prescriptions, and referrals), laboratory test results, and medications obtained from pharmacists (provided by the CatSalut database) (212). The SIDIAP study has been described in full previously (212, 213). Out of 318,020 individuals with T2D aged 30–99 years registered in the SIDIAP database in 2011, 62,002 had at least one measurement of ferritin. The final sample for the analysis included 38,617 individuals after excluding cases with missing values for covariates.

6.2.2 CVD events in the ET2DS and SIDIAP study

The ET2DS has data on prevalent and incident (over seven years) CVD events, and the SIDIAP study, on prevalent CVD only. Prevalent and incident events for ET2DS up to year 4 were collected using a combination of record linkage (hospital discharge and death certification data), repeat self-report and GP questionnaires, repeat ECG, and inspection of clinical notes. Incident events between year 4 and year 8 were collected using a combination of record linkage and inspection of clinical notes. Data linkage for ET2DS was undertaken by National Health Service National Services Scotland to the Scottish Morbidity Record (SMR01) and death records– general and acute inpatient discharge records using ICD-10 (www.who.int/classifications/icd/en/) (and related ICD-9 [www.icd9data.com/2007/Volume1]) codes. CVD cases of the SIDIAP study were defined on the basis of record linkage to hospital and death records.

Ischemic heart disease or CHD was defined in terms of primary diagnosis of angina or myocardial infarction (ICD-10 codes I20, I21, I22, I23). SIDIAP also included the category of "other acute ischaemic heart disease" (ICD-124). CEVD was defined in terms of stroke (ICD-10 codes I63, I64) and transient ischaemic attack (TIA) (ICD-10 code G45) as primary diagnoses. The ET2DS also included ICD-10 code I61 for intracerebral haemorrhage, and the SIDIAP study also considered the ICD-10 code G46 for vascular syndromes of brain in case of CEVD. In the ET2DS, fatal cases of myocardial infarction and stroke had the respective ICD-10 codes as primary cause of death. The ET2DS also used inspection of clinical notes to confirm CVD cases (prevalent or incident) if non-primary ICD-10 codes for CHD or CEVD were reported, except in the cases of angina.

6.2.3 Clinical examination and biochemical variables

The SIDIAP included clinical and biochemical variables similar to the ones in the ET2DS, with the exception of fibrinogen, CRP, and transaminases levels, and liver disease which were available in the ET2DS only. In the SIDIAP study, white blood cell count (WBC) was used as a measure of inflammation, and triglyceride levels were also available. Triglyceride levels, although also measured in the ET2DS, had

very few valid cases and thus this marker was not used as a covariate in the ET2DS analysis.

Systolic and diastolic brachial blood pressures were measured in the right arm to the nearest 2 mmHg with subjects in the supine position using a standard desk standing sphygmomanometer (Acceson™, AC Cossor & Son (Surgical) Ltd, Harlow, UK). Standing height was measured to the nearest mm, without shoes, using a wall-mounted vertical rule. Weight was assessed to the nearest 0.1 kg without outdoor clothing or shoes using SECA 761 electronic weighing scales. BMI was then calculated as weight (in kg) divided by the square of height (in m).

A questionnaire on smoking and alcohol consumption was self-completed at baseline in the ET2DS. If participants did not currently smoke or drink, they were asked about their smoking history and categorised as ever/never. This same categorisation was applied to the information collected on smoking and alcohol consumption in the SIDIAP system.

Fasting venous blood samples were taken to measure biochemical markers: triglycerides, HDL-C, total cholesterol, and creatinine by using enzymatic-colorimetric methods in both studies as well as fasting glucose in the ET2DS. In the ET2DS, GGT, AST, and ALT were measured by using the Elecsys 2010 electrochemiluminescence method (Roche Diagnostics, Burgess Hill, UK). CRP was determined using a high sensitivity immunonephelometric assay, and fibrinogen was measured in plasma anticoagulated with trisodium citrate using the automated Clauss assay (MDA-180 coagulometer, Organon Teknika). In both studies, glomerular fitration rate (eGFR) was calculated according to The Modification of Diet in Renal Disease (MDRD) equation using the serum creatinine values. In the SIDIAP study, WBC was carried out using coulter systems. In both studies, ferritin levels were measured by immunoturbidimetry (intra- and interassay coefficients of variation <8).

In the ET2DS, the liver disease was assessed by examining evidence of fatty infiltration using ultrasonography (Sieman's Medical Systems Inc, Washington, USA), performed by a single ultrasonographer who was unaware of the clinical and laboratory profiles of the subjects.

6.2.4 Data analyses for the ET2DS and SIDIAP study

Study variables were described as medians (interquartile ranges) for continuous variables or proportions for categorical variables. Ferritin levels were described using Z scores of the logarithm of ferritin values (i.e. as a continuous variable) and also as sex-specific quintiles (i.e. a categorical variable), in order to investigate potential linear and non-linear relationships. As mentioned previously in other chapters, the Z score of log-ferritin was chosen instead of ferritin or log-ferritin alone to facilitate the interpretation of effect estimates, in terms of changes in risk of the outcomes by changing standard deviations of the iron marker. As iron overload has been the most commonly reported factor for cardiometabolic risk in the general population, the lowest quintile of ferritin was used as the reference group. Baseline cross-sectional associations between ferritin and prevalent CVD were tested by using logistic regression. Longitudinal associations were evaluated by using Cox regression, and proportional hazards assumption was tested by the Schoenfeld residuals test and graphical method. For the analysis in the ET2DS, the years of follow-up were calculated from date of attendance at year 1 (baseline of this study) to the first of date of cardiovascular event, death, or end of December 2014. For individuals who developed both CHD and CEVD, the years of follow-up for incident or recurrent CVD were derived using the date of the first event during follow-up. Longitudinal associations were described by two approaches: 1) including people with prevalent CVD at baseline and adjusting for prevalent CVD, in order to have more statistical power and 2) excluding cases with prevalent CVD at baseline. In both cross-sectional and longitudinal analysis, the associations were described as unadjusted; adjusted for age and sex; and adjusted for age, sex, duration of diabetes, use of specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking (ever/never), alcohol consumption (ever/never), BMI, SBP and DBP, HbA1c, fasting glucose, HDL-C, total cholesterol, CRP and fibrinogen, liver enzymes, liver disease, and eGFR. All the above covariates were measured at year 1 of the ET2DS, the baseline for this analysis, with exception of age, CRP and fibrinogen, whose values were taken from the baseline of the original ET2DS, year zero. The cross-sectional analysis in the SIDIAP study included the above mentioned covariates except fibrinogen, CRP, fasting glucose, and transaminases levels, but

additionally included WBC and triglyceride levels. Liver enzyme levels, glucose, cholesterol, CRP, and duration of diabetes had skewed distributions and were log-transformed for the longitudinal analysis in the ET2DS. Skewed distribution of eGFR could not be approximated to normal distribution by any method of transformation and was used in its original form. The associations found did not differ whether untransformed or transformed variables were used in the analyses. The statistical tests were two-tailed to investigate associations with either low or high iron status. Further adjustment for anaemia (and haemoglobin levels), social class, and aspirin consumption were conducted to verify independent relationships where associations with iron status were found. A p value <0.05 was set as statistically significant. All the analyses were performed using STATA software 14.0. and SPSS 19.0.

6.3 Results

Three differences were observed in the study variables between included and excluded subjects in the ET2DS (Appendix Table 33). The people who were included had lower values of transaminase levels, HbA1C, and eGFR than the people who were excluded. In the SIDIAP study, the included group was older, had more women, longer duration of diabetes, and higher proportions of pharmacological treatments and CVD than the excluded group (Appendix Table 34). The subjects excluded had higher prevalence of smoking (ever) and alcohol intake (ever) and higher levels of all biochemical and clinical variables with the exception of HbA1C, SBP, and HDL-C for which there was no significant statistical difference between the groups.

Individuals in the ET2DS were younger, had higher ferritin levels, and higher proportion of subjects on lipid-lowering treatment and with history of smoking and alcohol consumption than those in the SIDIAP study (Appendix Table 35). The subjects from the SIDIAP study showed higher levels of DBP and serum total cholesterol and a larger proportion were treated with insulin (Appendix Table 35) than those from the ET2DS. At baseline, the prevalence of CHD, CEVD, and CVD was higher in the ET2DS than in the SIDIAP study (Appendix Table 35). In the

ET2DS, fasting glucose and inflammatory markers were also measured [median(interquartile range): glucose 6.4 (5.4–7.9) mmol/L; fibrinogen 3.5 (3.1–4.0) g/L; CRP 1.7 (0.8–4.0) mg/L; GGT 17 (10–31) U/L; ALT 31 (26–39) U/L; AST 29 (24–35) U/L]. Prevalence of liver disease was 1.5 % (n = 12) in the ET2DS. In the SIDIAP study, triglyceride levels [median (interquartile range): 1.4 (1.0–1.9) mmol/L], and white blood cells count (as inflammatory marker) [median(interquartile range): 7200 (6000–8500) cells/μL] were measured.

6.3.1 Cross-sectional associations

As expected, serum ferritin levels were significantly lower in women than in men in both the ET2DS [median, interquartile range 56 (23–100) μg/L vs. 96.5 (45.5–179.2) μg/L (P < 0.0001)] and the SIDIAP study [46.1(21–101) μg/L vs. 97.0 (39.0–208) μg/L (P < 0.0001)]. In both studies, CVD was more prevalent in men than in women [in the *ET2DS*: CVD, 41% vs. 28.1% (P < 0.001), CHD, 36.3% vs. 24.3% (P < 0.001), and CEVD, 10.9 vs. 6.3% (P = 0.018); in the *SIDIAP* study: CVD, 28.5 % v. 16.3% (P < 0.001), CHD, 22.0% vs. 11.4% (P<0.001), and CEVD, 9.1% vs. 5.9% (P < 0.001)].

SIDIAP study

The study variables of the 38,617 patients with T2D are described by sex-specific quintiles of ferritin in Table 17. The proportion of subjects reporting alcohol intake increased across the quintiles of serum ferritin. In this large population, the duration of diabetes and proportion of individuals taking oral anti-hypoglycaemic drugs decreased across quintiles of ferritin. The same pattern occurred with values of HbA_{1C}, SBP, and eGFR and with the proportion of cases with insulin, antihypertensive, and lipid-lowering treatments. There were positive dose-response relationships between serum ferritin concentration, serum total cholesterol, and triglyceride levels. There were higher proportions of people with CHD, CEVD, and CVD in the lower compared to the higher quintiles of ferritin (Table 17).

Among subjects from the SIDIAP study, ferritin, both as a continuous variable or in quintiles, was significantly inversely related to the likelihood of having CHD,

CEVD, or either diseases (Table 18). Similar findings were obtained in analyses stratified by sex (Appendix Table 36).

Table 17 Characteristics of people with type 2 diabetes by sex-specific quintiles of ferritin level in the SIDIAP study (n=38617)

	Ferritin (µg/L)					
	Q1 (n=7712)	**Q2(n=7740)**	**Q3(n=7724)**	**Q4(n=7732)**	**Q5(n=7709)**	**P for trend**
Sex Male/Female n	3283/4429	3275/4465	3296/4428	3290/4442	3279/4430	
Ferritin levels range by sex (µg/L)						
Men (n=14757)	*3.0-31.3*	*31.4-69.9*	*70.0-131.1*	*131.2-244*	*244.1-3507.8*	
Women (n=19847)	*1.0-17.7*	*17.8-34.0*	*34.1-62.0*	*62.1-121*	*121.1-3682.1*	
Variables						
Age (years)	74 (65-79)	75 (66-80)	74 (65-80)	72 (63-80)	71 (63-79)	<0.001
Duration of diabetes (years)	7.8 (5.4-11.5)	7.7 (5.2-11.1)	7.5 (4.6-10.8)	7 (3.6-9.9)	6.2 (2.6-8.8)	<0.001
Ferritin (µg/L)	13.3 (9.9-17)	31.6 (23.9-44.6)	57.6 (44.6-92)	110.1 (82-168.2)	273 (178.3-390.9)	<0.001
HbA1C (%)	7.1 (6.5-8)	7 (6.4-7.9)	6.9 (6.3-7.8)	6.8 (6.3-7.7)	6.8 (6.2-7.6)	<0.001
White blood cells count (cells/µL)	7.2 (6-8.5)	7.2 (6-8.5)	7.2 (6-8.6)	7.1 (6-8.5)	7.2 (6-8.6)	0.364
Smoking [ever]n(%)	2463 (31.9)	2202 (28.4)	2220 (28.7)	2274 (29.4)	2328 (30.2)	<0.001
Alcohol consumption [ever] n(%)	1338 (17.3)	1374 (17.8)	1567 (20.3)	1706 (22.1)	1897 (24.6)	<0.001
BMI (Kg/mts^2)	29.4 (26.5-33)	29.4 (26.3-32.8)	29.3 (26.4-32.9)	29.3 (26.4-32.9)	29.3 (26.3-32.9)	0.217
SBP mmHg	135 (125-142)	134 (125-141)	134 (125-141)	135 (125-141)	134 (125-140)	<0.001
DBP mmHg	73 (67-80)	73 (66-80)	74 (67-80)	73 (68-80)	75 (68-80)	<0.001
Total cholesterol mg/dl	4.52 (3.93-5.14)	4.57 (3.98-5.24)	4.62 (4.0-5.24)	4.68 (4.06-5.35)	4.73 (4.0-5.43)	<0.001
HDL-cholesterol mg/dl	1.21 (1.03-1.44)	1.21 (1.03-1.44)	1.24 (1.03-1.47)	1.24 (1.03-1.47)	1.21 (1.03-1.44)	0.403
Triglycerides mg/dl	1.43 (1.06-1.97)	1.43 (1.06-1.97)	1.41(1.03-1.96)	1.42 (1.05-1.97)	1.51 (1.10-2.10)	<0.001
eGFR, mL/min/1.73 m^2 *	60 (58.5-60)	60 (52.9-60)	60 (52.3-60)	60 (53.5-60)	60 (53.4-60)	<0.001*

	Ferritin (µg/L)					
	Q1 (n=7712)	**Q2(n=7740)**	**Q3(n=7724)**	**Q4(n=7732)**	**Q5(n=7709)**	**P for trend**
Oral hypoglycaemic drugs n(%)	6503 (84.3)	6185 (79.9)	5952 (77.1)	5575 (72.1)	5116 (66.4)	<0.001
Antihypertensive drugs n(%)	6312 (81.8)	6506 (84.1)	6284 (81.4)	6066 (78.5)	5896 (76.5)	<0.001
Insulin therapy n(%)	2026 (26.3)	1997 (25.8)	1808 (23.4)	1606 (20.8)	1378 (17.9)	<0.001
Lipid-lowering drugs n(%)	4961 (64.3)	4869 (62.9)	4944 (64)	4669 (60.4)	4327 (56.1)	<0.001
CHD n(%)	1504 (19.5)	1429 (18.5)	1296 (16.8)	1034 (134)	880 (11.4)	<0.001
CEVD n(%)	668 (8.7)	626 (8.1)	572 (7.4)	511 (6.6)	435 (5.6)	<0.001
CVD n(%)	2008 (26)	1894 (24.5)	1723 (22.3)	1441 (18.6)	1220 (15.8)	<0.001

Data are median (interquartile range) or *n* (%). * Values higher than 60 were recorded as 60. P for trend across quintiles by Jonckheere-Terpstra Test (continuous variables) and χ^2 tests (categorical variables). Q, quintile. CI, confidence interval. HDL-C, HDL cholesterol. BMI, body mass index. eGFR, estimate glomerular filtration rate. HbA1C, glycosylated haemoglobin. SBP, systolic blood pressure. DBP, diastolic blood pressure.*Negative trend is more evident in terms of rank values.

Table 18 Odds ratios and 95% confidence interval for prevalent cardiovascular disease by ferritin levels in the SIDIAP study (n=38,617)

	Model 1	Model 2	Model 3
CHD			
Ferritin (Z score of log-ferritin)	**0.76 (0.73-0.79) P<0.001**	**0.78 (0.75-0.81) P<0.001**	**0.83 (0.80-0.87) P<0.001**
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.93 (0.86-1.01)	0.89 (0.82-0.97)	0.91 (0.83-0.99)
Ferritin Quintile 3	**0.83 (0.76-0.90)**	**0.81 (0.74-0.88)**	**0.85 (0.77-0.93)**
Ferritin Quintile 4	**0.63 (0.58-0.69)**	**0.64 (0.59-0.70)**	**0.71 (0.64-0.78)**
Ferritin Quintile 5	**0.53 (0.48-0.58)**	**0.55 (0.50-0.60)**	**0.62 (0.56-0.69)**
P for trend	P<0.001	P<0.001	P<0.001
CEVD			
Ferritin (Z score of log-ferritin)	**0.88 (0.84-0.92) P<0.001**	**0.91 (0.86-0.95) P<0.001**	**0.92 (0.88-0.97) P=0.001**
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.92 (0.82-1.03)	**0.88 (0.78-0.99)**	**0.88 (0.78-0.99)**
Ferritin Quintile 3	**0.84 (0.75-0.94)**	**0.82 (0.73-0.92)**	**0.83 (0.73-0.93)**
Ferritin Quintile 4	**0.74 (0.66-0.84)**	**0.76 (0.67-0.85)**	**0.79 (0.70-0.89)**
Ferritin Quintile 5	**0.63 (0.55-0.71)**	**0.66 (0.58-0.75)**	**0.69 (0.60-0.78)**
P for trend	P<0.001	P<0.001	P<0.001
CVD			
Ferritin (Z score of log-ferritin)	**0.79 (0.76-0.82) P<0.001**	**0.82 (0.79-0.84) P<0.001**	**0.85 (0.83-0.88) P<0.001**
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	**0.92 (0.85-0.98)**	**0.87 (0.81-0.94)**	**0.88 (0.82-0.96)**
Ferritin Quintile 3	**0.81 (0.75-0.87)**	**0.79 (0.73-0.85)**	**0.81 (0.73-0.88)**
Ferritin Quintile 4	**0.65 (0.60-0.70)**	**0.65 (0.60-0.70)**	**0.71 (0.65-0.77)**
Ferritin Quintile 5	**0.53 (0.49-0.57)**	**0.54 (0.50-0.59)**	**0.61 (0.55-0.66)**
P for trend	P<0.001	P<0.001	P<0.001

CHD, coronary heart disease. CEVD, cerebrovascular disease. CVD, cardiovascular disease. Model 1: unadjusted. Model 2: adjusted for age and sex. Model 3: adjusted for adjusted for age, sex, duration of diabetes, use of specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking status, alcohol consumption, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, HDL-cholesterol, total cholesterol, white blood cells count, triglycerides level, and estimated glomerular filtration rate (eGFR). Significant associations are shown in bold (P<0.05).

ET2DS cohort

Study variables at baseline are described by sex-specific quintiles of ferritin in Table 19. Duration of diabetes and values of blood glucose, CRP, transaminases, and DBP increased across quintiles of ferritin. As in the SIDIAP study, there were higher proportions of individuals reporting alcohol consumption across increasing levels of ferritin, and use of oral anti-hyperglycaemic drugs, and duration of diabetes decreased across increasing levels of ferritin. CVD prevalence tended to decrease across ferritin quintiles (P for trend = 0.092) (Table 19).

Table 20 shows cross-sectional associations of ferritin with cardiovascular diseases at baseline. An inverse association of ferritin levels as continuous variable with CHD and any cardiovascular event was significant after adjustment for sex and age and remained significant after full adjustment. Analyses by sex showed that for women, but not for men, there was a significant inverse association between ferritin (highest vs. lowest quintile), CHD, and having any cardiovascular event after full adjustment [OR (95%CI) CHD 0.38 (0.15–0.97), P = 0.043; CVD: 0.40 (0.16–0.96), P = 0.041] (Appendix Table 37).

Table 19 Baseline characteristics of people with type 2 diabetes by sex -specific quintiles of ferritin level in the ET2DS (n=821)

	Q1 (n=162)	Q2(n=164)	Q3(n=167)	Q4(n=164)	Q5(n=164)	P for trend
Sex Male/Female n	84/78	85/79	85/82	84/80	84/80	
Ferritin levels range by sex (µg/L)						
Men (n=422)	7-36	37-74	75-123	124-198	199-1095	
Women (n=399)	5-20	21-38	39-69	70-119	120-605	
Variables						
Age (years)	69.9(65.5-72.7)	68.4(65.2-71.5)	68.6(65.2-72.5)	68.9(64.8-72.1)	69.7(65.5-72.8)	0.751
Duration of diabetes (years)	9(5-14)	8(4-13)	8(5-11)	7(4-10)	5(3-10)	<0.001
Ferritin (µg/L)	17(11-25.2)	38(28-52)	75(55-98)	124(87-162.5)	246(177.5-338)	<0.001
Glucose mmol/L	6.2(5.0-8.0)	6.5(5.5-7.9)	6.3(5.3-7.5)	6.4(5.4-7.9)	6.7(5.8-8.2)	0.011
HbA1C (%)	7.0(6.4-7.9)	7.0(6.5-7.7)	7.0(6.4-7.5)	7.0(6.4-7.7)	7.0(6.4-7.8)	0.525
Fibrinogen (g/L)	3.5(3.1-4.0)	3.7(3.1-4.1)	3.6(3.0-4.21)	3.5(3.1-3.9)	3.5(3.1-4.0)	0.781
CRP(mg/L)	1.5(0.7-3.0)	1.5(0.7-3.9)	1.8(0.8-4.6)	2.1(1.1-4.3)	2.2(1.0-4.5)	<0.001
GGT(U/L)	13(8-25.5)	14(9.0-23)	17(10-29)	17.5(10-31.7)	24.5(13-44.7)	<0.001
ALT(U/L)	29(24-34)	28(25-36)	31(25-36)	34(27.2-41)	36(28-47)	<0.001
AST(U/L)	27(22-32)	27(24-32)	28(24-33)	29(25-34.7)	33(27-41)	<0.001
Smoking [ever] n(%)	101(62.3)	89(54.3)	101(60.5)	103(62.8)	96(58.5)	
Alcohol consumption [ever] n(%)	124(76.5)	129(78.7)	143(85.6)	136(82.9)	141(86)	<0.001
BMI (Kg/mts^2)	30.8(27.6-34.4)	30.5(26.7-34.1)	30.2(27.3-33.8)	30.6(27.1-35.4)	30.7(27.9-34.0)	0.485
SBP mmHg	132(120-142)	132(122-142)	130(122-140)	133(120-142)	134(124-144)	0.685
DBP mmHg	68(60-74)	68(62-74)	70(62-76)	70(64-76)	70(64-78)	0.031
HDL-cholesterol (mmol/L)	1.3(1.1-1.5)	1.3(1.1-1.5)	1.2(1.1-1.4)	1.2(1.0-1.4)	1.2(1.0-1.5)	0.006
Total cholesterol (mmol/L)	4.1(3.7-4.6)	4.2(3.8-4.7)	4.1(3.6-4.8)	4.3(3.8-4.9)	4.2(3.7-4.8)	0.166
eGFR, mL/min/1.73 m^2	60(60-65.2)	60(60-71)	60(60-72)	60(60-73)	60(60-68.5)	0.757
Oral hypoglycaemic drugs n(%)	137(84)	134(81.7)	124(74.3)	119(72.6)	93(56.7)	<0.001
Antihypertensive drugs n(%)	145(89.5)	139(84.8)	147(88)	136(82.9)	138(84.1)	0.147

	Q1 (n=162)	Q2(n=164)	Q3(n=167)	Q4(n=164)	Q5(n=164)	P for trend
Lipid-lowering drugs n(%)	147(90.7)	132(80.5)	139(83.2)	137(83.5)	136(82.9)	0.168
Insulin therapy n(%)	28(17.3)	25(15.2)	27(16.2)	21(12.8)	22(132.4)	0.250
Liver disease n(%)	2(1.2)	0(0)	1(0.6)	6(3.7)	3(1.8)	0.102
Outcomes at baseline n(%)						
CHD	59(36.4)	48(29.3)	50(29.9)	49(29.9)	44(26.8)	0.104
CEVD	15(9.3)	15(9.1)	12(7.2)	19(11.6)	10(6.1)	0.576
CVD (any cardiovascular event)	67(41.4)	58(35.4)	52(31.1)	55(33.5)	53(32.3)	0.092
Outcomes after follow-up						
Development of CHD	21(13)	12(7.3)	16(9.6)	20(12.2)	9(5.5)	0.107
Development of CEVD	17(10.5)	7(4.3)	10(6.0)	9(5.5)	11(6.7)	0.306
Development of CVD	35(21.6)	17(10.4)	24(14.4)	27(16.5)	20(12.2)	0.168

Data are median (interquartile range) or *n*(%). P for trend across quintiles by Jonckheere-Terpstra Test (continuous variables) and χ^2 tests (categorical variables). Q, quintile. CI, confidence interval. HDL-C, HDL cholesterol.. BMI, body mass index. GGT, gamma-glutamyl transpeptidase. ALT, Alanine aminotransferase. AST, Aspartate aminotransferase. CRP, C reactive protein. eGFR, estimate glomerular filtration rate. HbA1C, glycosylated haemoglobin. SBP, systolic blood pressure. DBP, diastolic blood pressure.

Table 20 Odds ratios and 95% CI for prevalent cardiovascular events by ferritin levels in the ET2DS (n=821)

	Model 1	Model 2	Model 3
CHD			
Ferritin (Z score of log-ferritin)	0.93(0.80-1.08) P=0.367	**0.84(0.72-0.99) P=0.039**	**0.81(0.67-0.97) P=0.029**
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.72(0.45-1.14)	0.74(0.46-1.19)	0.82(0.49-1.37)
Ferritin Quintile 3	0.74(0.47-1.18)	0.76(0.48-1.22)	0.64(0.38-1.08)
Ferritin Quintile 4	0.74(0.46-1.18)	0.76(0.47-1.21)	0.76(0.44-1.29)
Ferritin Quintile 5	0.64(0.39-1.02)	0.63(0.39-1.02)	0.61(0.35-1.06)
P for trend	0.104	0.103	0.094
CEVD			
Ferritin (Z score of log-ferritin)	0.97(0.76-1.24) P=0.862	0.89(0.69-1.14) P=0.377	95(0.71-1.28) P=0.768
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.98(0.46-2.09)	0.98(0.46-2.09)	1.09(0.49-2.39)
Ferritin Quintile 3	0.75(0.34-1.67)	0.76(0.34-1.68)	0.69(0.30-1.60)
Ferritin Quintile 4	1.28(0.62-2.62)	1.28(062-2.64)	1.44(0.66-3.15)
Ferritin Quintile 5	0.63(0.27-1.46)	063(0.27-1.46)	0.77(0.31-1.92)
P for trend	0.576	0.583	0.926
CVD			
Ferritin (Z score of log-ferritin)	0.93(0.80-1.07) P=0.357	**0.84(0.72-0.98) P=0.033**	**0.80(0.67-0.96) P=0.020**
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.77(0.49-1.21)	0.79(0.50-1.25)	0.88(0.54-1.45)
Ferritin Quintile 3	0.64(0.40-1.00)	0.65(0.41-1.03)	0.53(0.32-0.88)
Ferritin Quintile 4	0.71(0.45-1.12)	0.72(0.46-1.14)	0.70(0.41-1.17)
Ferritin Quintile 5	0.67(0.43-1.06)	0.67(0.42-1.06)	0.64(0.37-1.10)
P for trend	0.093	0.091	0.066

CI, confidence interval. CHD, coronary heart disease. CEVD, cerebrovascular disease. CVD, cardiovascular disease. Model 1: unadjusted. Model 2: adjusted for age and sex. Model 3: adjusted for adjusted for age, sex, duration of diabetes, use specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking status, alcohol consumption, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, fasting glucose, HDL-cholesterol, total cholesterol, C reactive protein (CRP), fibrinogen, transferases levels, liver disease and estimated glomerular filtration rate (eGFR) at baseline. Significant associations are shown in bold (P<0.05).

Longitudinal findings (ET2DS)

After a mean follow-up of 6.1 ± 1.7 years (median, 6.7; interquartile range, 6.3–6.9), the number (proportion) of people with incident or recurrent outcomes was as follows: 123 (15%) with either CHD and/or CEVD, 78 (9.5%) with CHD, and 54 (6.6%) with CEVD. Nine people experienced both types of events during the follow-up. Among participants without disease at baseline (n = 536) with a mean follow-up of 6.2 ± 1.5 years [median 6.7 (6.4–7.0)], there were 60 participants (11.2%) with incident CVD, 38 (7.1%) with incident CHD, and 25 (4.7%) with incident CEVD. There was no significant difference by sex in the number of incident or recurrent cases for CVD (57 men, 66 women), CHD (34 men, 38 women), or CEVD (32 men, 22 women) during follow-up. There were 132 (16.1%) deaths during the follow-up, with 26 of the deaths (19.6%) due to CVD, of which 19 were attributable to CHD.

HRs and 95%CI for incident/recurrent CVD are shown in Table 21 for both standardised values and quintiles of ferritin. When comparing the extreme quintiles, ferritin was inversely associated with development of CHD and CVD in unadjusted, partially adjusted, and fully adjusted models [Fully adjusted HR (95%CI) CHD: 0.39 (0.16–0.93), P = 0.035; any cardiovascular event: 0.50 (0.27–0.88), P = 0.044]. Moreover, further adjustment for prevalent CVD at baseline (model 4) did not affect the estimates and significance of the association [CHD: 0.39 (0.16–0.94), P = 0.036; any cardiovascular event: 0.50 (0.27–0.94), P = 0.044] (Table 21). There were no significant associations when ferritin was included in models as a continuous variable. When restricting the analyses to 536 people without prevalent CVD at baseline (Appendix Table 38), the longitudinal inverse associations between extreme quintiles for CHD and CVD was observed again [model 1, P = 0.016; model 2, P = 0.017] although the associations had a marginal significance in the fully adjusted model [CHD: 0.28 (0.07–1.14), P = 0.078; CVD: 0.42 (0.16–1.08), P = 0.074] (Appendix Table 38), presumably owing to lower statistical power.

I considered additional potential confounders influencing the inverse ferritin-cardiometabolic risk association in the ET2DS and SIDIAP study. In the ET2DS, almost half of the subjects reported having higher income occupations (managerial,

administrative, and professional), 67.1% underwent treatment with aspirin, and prevalence of anaemia was 11.8% (97/814, 7 missing values) although only the distribution of anaemia varied significantly by ferritin quintiles (prevalence 26.1 % in quintile 1 and 6.1 % in quintile 5, χ^2 test P > 0.001). In the SIDIAP study, 43.8% of patients reported receiving antiplatelet agents and 39.9% had anaemia, and the distribution of these variables significantly decreased across ferritin quintiles (Antiplatelet agents 51.6% in quintile 1 and 32.8% in quintile 5, χ^2 test P > 0.001; Anaemia 59.9 % in quintile 1 and 31.4 % in quintile 5, χ^2 test P > 0.001).

The inverse associations between ferritin and CVD in the ET2DS and the SIDIAP study did not substantially change after further adjustment for the above covariates (P < 0.05). The same occurred when individuals with extremely high serum ferritin levels were excluded (>300 µg/L, n = 54 in ET2DS and n = 3,239 in SIDIAP study). After excluding individuals with iron deficiency in the ET2DS (ferritin<15 µg/L, n=72), the association of ferritin with prevalent CHD was weakened presumably due to lower statistical power [fully adjusted HR (95%CI) 0.84(0.66-1.07), P=0.179], but the association with incident/recurrent CHD remained similar [ferritin highest v. lowest quintile fully adjusted HR (95%CI) 0.37(0.14-0.97), P= 0.045]. On the other hand, the inverse association between higher ferritin (highest v. lowest quintile) and incident CHD in subjects with no prevalent CVD at baseline reached statistical significance and the association was slightly strengthened [HR (95%CI) 0.22(0.05-0.95), P= 0.043]. However, this might be a chance finding since it was not consistent with the above effects of removing individuals with iron deficiency in a larger sample. No other substantial changes in effect estimates or statistical significance were observed when individuals with iron deficiency were removed. In addition, I performed an analysis limited to individuals aged 60–75 years of age from the SIDIAP study (n = 16,491), to confirm comparability of age group with the ET2DS cohort, and the associations between ferritin, CHD, CVED, and CVD remained similar. The same was true for associations between ferritin, CHD, and CVD in individuals younger than 60 years (n = 5,857). There was no evidence of a J or U-shape association on comparing extreme quintiles and middle quintiles together (2, 3, and 4) or using quintile 3 as the reference group.

Despite the differences previously described between included and excluded individuals in the SIDIAP study, the inverse association between ferritin and CVD outcomes in the excluded individuals was also statistically significant. To test the above, I used partial models of adjustment (age, sex, duration of diabetes, and one more covariate at the time) since a total adjustment was not feasible owing to missing values in one or more covariates in the excluded subjects (data not shown).

Table 21 HRs and 95% CI for incident/ recurrent cardiovascular events by ferritin levels in the ET2DS (n=821)

	Model 1	Model 2	Model 3	Model 4
CHD				
Ferritin (Z score of log-ferritin)	0.88(0.71-1.10) P=0.286	0.87(0.69-1.09) P=0.241	0.90(0.68-1.17) P=0.440	0.91(0.69-1.18) P=0.491
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.52(0.25-1.06)	0.54(0.26-1.10)	0.63(0.30-1.31)	0.63(0.30-1.31)
Ferritin Quintile 3	0.70(0.36-1.34)	0.71(0.37-1.37)	0.68(0.34-1.37)	0.70(0.35-1.42)
Ferritin Quintile 4	0.88(0.48-1.64)	0.91(0.49-169)	0.95(0.48-1.86)	0.97(0.49-1.92)
Ferritin Quintile 5	**0.38(0.17-0.84)**	**0.38(0.17-0.83)**	**0.39(0.16-0.93)**	**0.39(0.16-0.94)**
P for trend	0.132	0.122	0.170	0.186
CEVD				
Ferritin (Z score of log-ferritin)	0.87(0.66-1.13) P=0.319	0.81(0.61-1.07) P=0.142	0.85(0.62-1.16) P=0.325	0.85(0.62-1.17) P=0.334
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	**0.37(0.15-0.90)**	**0.38(0.15-0.92)**	0.54(0.21-1.35)	0.54(0.21-1.34)
Ferritin Quintile 3	0.52(0.24-1.15)	0.53(0.24-1.17)	0.55(0.24-1.25)	0.56(0.24-1.28)
Ferritin Quintile 4	0.47(0.21-1.06)	0.48(0.21-1.07)	0.57(0.23-1.38)	0.58(0.24-1.40)
Ferritin Quintile 5	0.58(0.27-1.25)	0.57(0.27-1.23)	0.66(0.27-1.58)	0.66(0.27-1.59)
P for trend	0.236	0.218	0.344	0.351
CVD				
Ferritin (Z score of log-ferritin)	0.89(0.74-1.06) P=0.199	0.86(0.71-1.03) P=0.112	0.87(0.71-1.07) P=0.211	0.88(0.71-1.08) P=0.236
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	**0.43(0.24-0.77)**	**0.44(0.25-0.79)**	**0.54(0.29-0.98)**	**0.54(0.29-0.98)**
Ferritin Quintile 3	0.61(0.36-1.03)	0.62(0.37-1.05)	0.62(0.35-1.07)	0.64(0.37-1.11)
Ferritin Quintile 4	0.70(0.42-1.16)	0.72(0.43-1.19)	0.78(0.45-1.35)	0.80(0.46-1.38)
Ferritin Quintile 5	**0.50(0.29-0.87)**	**0.49(0.29-0.86)**	**0.50(0.27-0.94)**	**0.50(0.27-0.94)**
P for trend	0.104	0.093	0.116	0.123

HR, hazard ratio. CI, confidence interval. CHD, coronary heart disease. CEVD, cerebrovascular disease. CVD, cardiovascular disease. Model 1: unadjusted. Model 2: adjusted for age and sex. Model 3: adjusted for adjusted for age, sex, duration of diabetes, use specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking status, alcohol consumption, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, fasting glucose, HDL-cholesterol, total cholesterol, C reactive protein (CRP), fibrinogen, transferases levels, liver disease and estimated glomerular filtration rate (eGFR) at baseline. Model 4: Model 3 plus prevalent cardiovascular disease at baseline. Values of transaminases levels, glucose, cholesterol, CRP and duration of diabetes were log-transformed for the analysis. Significant associations are shown in bold (P<0.05).

6.4 Discussion

This chapter described independent, inverse cross-sectional, and prospective associations between ferritin levels and CVD in representative samples of people with T2D from Catalonia, Spain and from Scotland. The multivariate adjusted estimates are similar to the previous unadjusted cross-sectional observations by Hermans et al. (210), suggesting that the inverse association between ferritin levels and incident or recurrent CVD in people with T2D is also observed in larger samples in a prospective design and in populations at different levels of cardiovascular risk. This pattern differs from the pattern of iron excess and cardiometabolic risk commonly described in the general population. However, paradoxically, ferritin in both studies also presented positive cross-sectional associations with cardiovascular risk factors such as fasting glucose and diastolic blood pressure and negative association with HDL-C, as observed in general populations.

6.4.1 Comparison with other studies

As mentioned above, there are few other studies to which I can compare the findings in this chapter. Besides the cross-sectional evaluation by Hermans et al. (210), two studies investigated the iron status among people with vascular complications of diabetes, and their aims, outcomes, or design were different from those of the present study. In one of them, iron depletion by phlebotomy in people with diabetes and peripheral arterial disease (n = 636, controls 641) was not found to affect all-cause mortality or incidence of cardiovascular events (214). In the other one, both low and high ferritin levels, and high sTfR levels were associated with 5-year all-cause mortality rate in 287 people with diabetes and stable coronary artery disease (29). My results are similar in part to those of this latter study, in terms of a pattern of low iron status (low ferritin, high sTfR) being associated with poor prognosis in people with T2D, although I did not observe U or J-shaped associations. A nested case-control study (n = 60 cases of myocardial infarction; 112 controls) from the Rotterdam Study cohort reported marginal association between high ferritin (highest tertile) and myocardial infarction with stronger associations in subgroups of smokers and people with diabetes (215). However, the very small sample size in those sub-

groups (n = 34 subjects with diabetes) along with multiple testing did not provide conclusive evidence of the association.

6.4.2 Cross-sectional and prospective associations – Inverse but slightly different

The cross-sectional association of prevalent CVD with ferritin levels showed a clear inverse pattern in the ET2DS and SIDIAP study. In the ET2DS, the inverse cross-sectional ferritin-CVD association was mainly based on CHD and the association in women, whereas in the SIDIAP study, there were associations of ferritin with all cardiovascular outcomes in both sexes. In the ET2DS, although the longitudinal inverse ferritin-incident/recurrent CVD association did not show a linear pattern as seen in the cross-sectional analysis of the same study, an inverse association between iron status and incident/recurrent CVD was observed when comparing extreme quintiles. Low iron status could explain the higher cardiovascular risk, since the ORs were lower than one across increasing quintiles of ferritin when compared with the lowest quintile of ferritin, which reflects iron deficiency. Although iron excess has been the most reported iron imbalance associated with IR and development of diabetes and MetS in general populations, its association with cardiovascular disease is still controversial. Iron excess reflected in high ferritin levels was positively associated with incident CHD in Finnish men but subsequent studies failed to replicate this association (157). In line with the findings of this chapter, a significant negative association between transferrin saturation and development of coronary artery disease or myocardial infarction has been identified in the general population in a recent meta-analysis, leading to the conclusion that high body iron stores could confer protection against development of CHD (183). However, as mentioned in the discussion in Chapter 5, in that meta-analysis, no significant association was found between serum ferritin, total iron-binding capacity, serum iron, and CHD. Similarly, recent research has been focused on anaemia/iron deficiency as a frequent comorbidity of coronary artery disease and heart failure (216).

6.4.3 Adjustment model and its lack of effects on the associations

Since I found an inverse association between iron status and development of CHD, it is not surprising that covariates of body mass and markers of liver injury, glucose metabolism, and inflammation did not substantially attenuate the association. In general, these covariates are used as confounding factors for positive associations since they are related to increasing ferritin levels and higher cardiometabolic risk. The pattern of lower iron status and higher cardiovascular risk in people affected by T2D appears to be similar to the case of lower BMI and higher risk of diseases and death in certain populations, a phenomenon knowns as the obesity paradox. In people with T2D and cardiovascular complications, a modestly higher BMI is associated with lower risk of death(217). There is not a clear explanation for this phenomenon, although residual confounding from inaccurate measurement of smoking status, excessive anticoagulation and co-morbidity has been mentioned as factors to be considered(217).

6.4.4 Implications of additional adjustments and controversy about the association between iron status and CVD

Given the direction of the associations found, I evaluated additional covariates that might contribute to increased CVD risk across quintiles 1–5 of ferritin levels. Anaemia has been previously found to be a predictor of CHD among 14,410 individuals (218). Ventricular remodelling, cardiac dysfunction, or myocardial ischemia could be an underlying mechanism (218). There have also been reports on decreased peripheral endothelial progenitor cells with impaired function and vulnerable plaque components in patients with acute coronary syndrome and anaemia (219). Anaemia is a common finding in patients with diabetes owing to the high burden of renal complications in this population (220). However, anaemia or haemoglobin levels did not substantially affect the inverse association of ferritin with prevalent or incident recurrent CHD in the studies reported in this chapter. This can be interpreted as follows: 1) low iron stores either with and without progression to impaired erythropoiesis are associated with prevalent cardiovascular complications in people with T2D, and deleterious effects of anaemia on vascular function are not

mediating that association, and 2) normal haemoglobin status is not the cause of an apparent protective effect of high ferritin for lower risk of incident CHD and CVD. The above findings support previous observations, indicating that iron deficiency is not the main cause of anaemia and, if present, it does not necessarily lead to anaemia in people with diabetes (221).

Aspirin consumption is well known for promoting iron loss by occult bleeding, and low serum ferritin could be linked to the anti-inflammatory action of aspirin on cytokine-induced ferritin production in people with inflammatory conditions (222). Hermans et al., in their cross-sectional study, did not find significant differences in ferritin levels between groups of people reporting aspirin consumption, but they did not perform any statistical adjustment for aspirin intake (210). In the present analyses, aspirin treatment had no influence on the inverse association between ferritin and CHD/CVD in full multivariable models. In some populations, low income or deprived sub-groups may have low body iron stores (223) and high rates of chronic diseases due to malnutrition (224). However, adjustment for social class did not affect the cross-sectional and longitudinal associations described in this chapter.

6.4.5 CEVD and ferritin levels

Ferritin was associated with CEVD in people with T2D from the SIDIAP study only. There are only a few studies and conflicting findings on the association between serum ferritin and stroke or CEVD in the general population as discussed in Chapter 5. In people affected by T2D, only the study by Hermans et al. showed that stroke was more common in men with diabetes and higher ferritin (highest quartile vs. the lowest). Future studies should focus on different kinds of CEVD in people with T2D for better characterisation of the relationship between iron and CVD. The ferritin-CEVD association may require a large sample size to give adequate statistical power since this was only observed in the large population of the SIDIAP study.

6.4.6 High iron status: Protective factor?

The inverse association between iron status and CVD also suggests that high iron status is a protective factor against CVD in people with diabetes. Hermans et al. hypothesised this by highlighting the lesser known pleiotropic anti-oxidant properties of ferritin itself, potentially acting in a compensatory response to inflammation in T2D (210). Findings in murine models of oxidative stress-induced synthesis of iron proteins in diverse cell lines support this idea since inflammation is linked to increased oxidative stress (225). However, in the present study, the association was unaffected when adjusting for inflammatory markers such as CRP or fibrinogen levels.

The findings may also be related to pleiotropic effects of ferritin on blood vessel formation. An *in vitro* study showed that ferritin stimulates proliferation of endothelial cells via inactivation of HKa, which is a potent angiogenesis inhibitor (226). Therefore, a higher iron status in T2D, in terms of high ferritin, might preserve the integrity of vascular tissue, decreasing the risk of cardiovascular injuries.

High iron status as a protective factor for cardiometabolic risk in T2D could also be related to the course of T2D rather than the direct causal beneficial effects of ferritin. Systemic metabolic alterations triggered by T2D can encompass several regulatory pathways of iron metabolism affecting ferritin levels, circulating iron, and ultimately the cardiovascular risk attributable to iron. A recent study in mouse and *in-vitro* models confirmed that starvation leads to increased serum ferritin levels, hepatic and splenic iron retention, and decreased serum iron via gluconeogenesis, a persistently active pathway in T2D, with a subsequent increase in the levels of the hormone hepcidin (227). In other words, high ferritin levels in T2D could reflect circulatory iron depletion which would help avoid the pro-oxidant effects of iron on vascular function. It is also likely that T2D influences iron proteins through other mechanisms and/or that there is residual confounding from factors not measured in the present study.

An increased level of circulating ferritin, as a mechanism to prevent oxidative damage, might be another plausible explanation for the inverse association between serum ferritin and CVD in T2D patients. Ferritin levels may have a dual role with regard to free iron and its harmfulness. High serum ferritin indicates high body iron accumulation, but these raised levels of the protein are also an attempt to catch the free iron and prevent oxidative damage. It can be assumed that there is residual free iron in iron overload despite very high levels of ferritin. Therefore, low ferritin levels may predispose one to oxidative damage by free iron if there is down-regulation of ferritin synthesis in T2D despite normal or high iron intake.

6.4.7 Consistency of the association in two different populations

Scotland and Catalonia have very different cardiovascular risk profiles even among people with T2D as illustrated by the higher prevalence of CVD in the ET2DS than in the SIDIAP populations. Scotland has one of the highest CHD mortality rates in Europe, and the decline in CHD mortality since 1994 now appears to be slowing in young adults (228, 229). Moreover, the prevalence of well-known CVD risk factors increased between 1995 and 2008 in the Scottish population (229). The prevalence of self-reported diabetes increased by 0.12% per year, and the prevalence of self-reported hypertension was 4.3% higher in 2008 than in 1995 (229). Lower rates of coronary events were noted in the Catalan population than in the Scottish populations in the MONICA study, which compared risk factor prevalence and CVD outcomes among 21 countries during 10-year follow-up from early 1980's till early 1990's (230, 231). Lifestyle, mostly Mediterranean diet, seems to offer cardiovascular benefits in term of better lipid profiles, insulin sensitivity, and blood pressure, with concomitantly reduced CVD risk (232). Despite the differences mentioned above, the association between low ferritin and higher CVD risk was consistent in T2D patients in the Scottish and Catalan populations analysed in this chapter, mainly on the basis of CHD. Thus, geographical and cultural differences may not influence the iron-cardiometabolic risk relationship in people with T2D. However, further studies in diverse populations are needed to confirm this hypothesis.

6.4.8 Paradox: Inverse association with established CVD but positive with cardiovascular risk factors

It is important to highlight that there appears to be a coexistence of both iron excess and low iron status patterns with regard to cardiometabolic risk factors in people with T2D. In the analyses of this chapter, cross-sectional positive dose-response relationships of serum ferritin were observed with glucose, triglycerides, and blood pressure. Similarly, Hermans et al. found lower prevalence of cardiovascular complications but higher IR index in their population of people with diabetes with higher serum ferritin levels. However, it is unclear why there was no consistent pattern in the relationship between ferritin and CVD and its risk factors. Survival bias may be a possible explanation if cases with high ferritin had died prior to having the opportunity to join the ET2DS cohort. Future studies should also focus on longitudinal associations of iron markers and risk factors of CVD in people affected by T2D to examine if the conflicting pattern persists over time.

6.4.9 Strengths and weaknesses

To the best of my knowledge, the ET2DS is the first longitudinal study in which it has been possible to describe the association between body iron stores and cardiovascular complications in T2D patients. Additional strengths of this study included the use of large and representative samples of people with T2D and a broad set of covariates in an attempt to identify an independent association.

CRP, haemoglobin, and fibrinogen levels were measured at the original baseline of the ET2DS, one year before the ferritin levels were measured. However, CRP and haemoglobin levels were related in a dose-response manner to ferritin quintiles, in line with the positive associations between these markers as observed in diverse populations (233, 234), although with variable and modest strength in the case of CRP (26, 140, 235).

There were slight differences in definitions of CHD and CEVD between the ET2DS and the SIDIAP studies owing to the predetermined ways of establishing the disease

variables in each study. This may have led to biased findings on the ferritin-CEVD association, which was significant in the SIDIAP study only, but as mentioned previously, this discrepancy could also be related to the greater statistical power of the SIDIAP study. On the other hand, the inverse ferritin-CHD association was consistent in both studies.

There is potential for selection bias in the SIDIAP cohort because serum ferritin measurement was probably performed in subjects suspected of having anaemia and iron disorders. Because of this, only around 10 % of the original SIDIAP sample could be used in the study. As mentioned in Chapter 5, multiple imputation techniques may help to deal with potential bias derived from missing values. The same disadvantages or limitations to impute data described in Chapter 5, are also applicable to this analysis. It is important to take into account that adjustment for anaemia did not affect the significance of the associations in the SIDIAP analysis. In addition, although excluded individuals (those with incomplete data) had different characteristics from those who were included in the analysis, the association between ferritin and CVD was present in included and excluded individuals (in partial models in the latter group due to missing data). Major selection bias in the SIDIAP study seems unlikely given that the ET2DS showed similar patterns of associations. There was also a difference in the age ranges of the studies evaluated, but the sensitivity analysis using identical age ranges did not alter the findings. Data regarding oxidative stress markers were not available and these would allow more robust adjustments and better inferences about the probable underlying mechanisms. The findings of this analysis should not be generalised before replication in other populations with T2D, in which iron status and distribution of covariates could be different.

6.5 Conclusion

In this chapter, serum ferritin levels were inversely associated with prevalent, incident, and recurrent CVD in people with T2D. Although the underlying mechanisms behind this association remain unclear and further studies of representative populations are required, subclinical/clinical inflammation, BMI, lipid levels, concomitant treatment, markers of liver injury, glucose metabolism markers,

anaemia, aspirin intake, and duration of diabetes do not appear to be the primary explanatory variables. Iron deficiency seems to be harmful for cardiovascular health in the case of T2D and high iron status could play a protective role. However, it is also plausible that T2D could selectively influence iron proteins through unknown mechanisms and that residual confounding could have contributed to the association. As recently proposed, whether replenishing iron stores in patients with coronary artery disease and iron deficiency is beneficial or not cannot yet be answered with certainty (157).

The next chapter will summarise and contrast the findings throughout all the chapters of this PhD thesis. General conclusions and targets for future research will be also discussed.

Chapter 7

Summary and conclusions

The aim of this chapter is to summarise the findings of this doctoral thesis and to discuss them within the context of the overall reflections and recommendations emerging from this research study. More detailed discussions regarding the analyses conducted have been presented in each specific chapter.

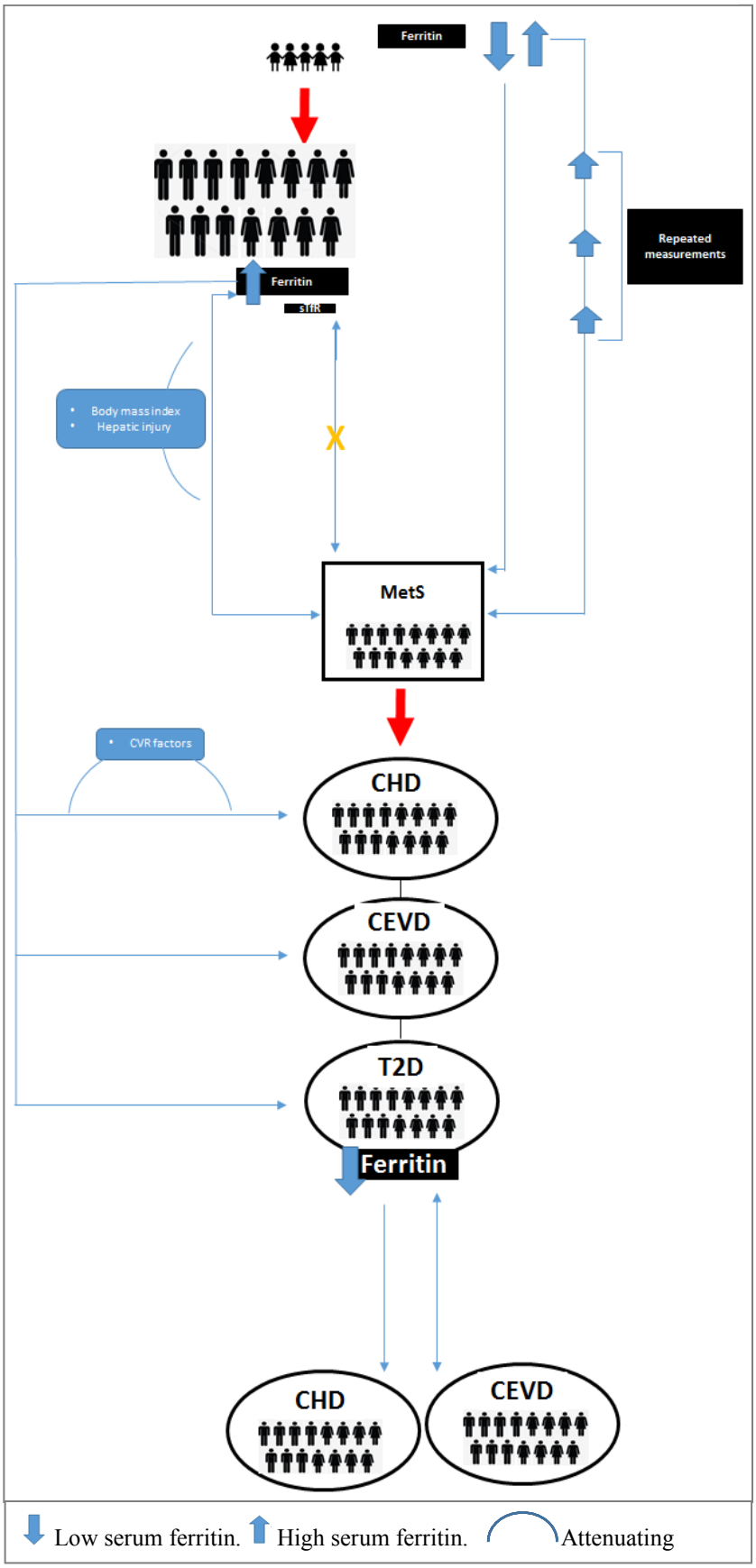
7.1 Summary

Figure 10 provides a summary of the findings of this PhD thesis using the same scheme as in Figure 1 of the introductory chapter which showed the relationships and gaps to be explored.

Chapter 1 presented a review of empirical research literature on the relationship between iron metabolism and cardiometabolic risk, concepts surrounding iron markers, and the study outcomes. This chapter also described the gaps in the understanding of the iron-cardiometabolic risk relationship, which were subsequently explored in Chapters 2 to 6.

Chapter 2 focused on the first research question, which aimed to explore the link between serum ferritin and transferrin and MetS in children and adolescents. The association between serum ferritin and MetS has been investigated in many adult populations, but not in the early stages of life. The empirical analyses showed that iron stores, as reflected by ferritin levels, can be either positively or negatively associated with MetS and glucose metabolism markers in different paediatric populations. Furthermore, sustained high ferritin levels at various time points and increases in ferritin levels during childhood were associated with higher MetS score at adolescence.

Figure 10 Summary of associations identified between iron markers and cardiometabolic outcomes across populations of children, adults, and people with T2D evaluated in this thesis.



The third chapter described the association between serum ferritin status and MetS in adults. No previous studies had evaluated this relationship in Scottish populations; therefore, data from two populations from this country were analysed. This chapter also looked at the overall association between ferritin, MetS, and each MetS component in adults, by conducting a meta-analysis and investigating potential relevant sources of heterogeneity in the association. Interestingly, ferritin levels in the Scottish population were positively associated with MetS, but the association was not independent of the effect of covariates, mainly BMI and transaminase levels. The meta-analysis supported this finding by describing hepatic injury markers and BMI as the major attenuating factors in the ferritin-MetS association. This chapter also described how the association may differ depending on the distribution of potential confounding factors in certain populations and that high fasting glucose and triglycerides are the MetS components most strongly associated with high serum ferritin levels.

Chapter 4 investigated the association between iron status and MetS in adults using a serum marker of tissue iron demand – sTfR. The sTfR-MetS association had been examined in a single previous study with negative results. The analysis I conducted in a Croatian population did not show an association between sTfR and MetS either. In contrast, serum ferritin was positively associated with MetS in this population independently of covariates. These contrasting results suggest that different iron markers may reflect different physiological processes other than iron metabolism.

Chapter 5 evaluated the longitudinal association between serum ferritin and several CMDs in the same population to extend the earlier work on the association between ferritin and CMD risk factors. Samples of the nationally representative Scottish Health Surveys (SHeS) 1995 and 1998 were used for this purpose. According to previous literature, the association between ferritin and different CMDs is heterogeneous. There is a well-established positive association with T2D, conflicting findings regarding the association with CHD, and sparse studies with inconclusive evidence on the association with CEVD. The analyses I conducted showed a positive longitudinal association between higher ferritin levels and CEVD, which was strengthened by using higher cut-points for increased ferritin levels and an

association with CHD in unadjusted analyses. My analyses confirmed the widely established association of ferritin with T2D, even with serum ferritin within the normal range. The above set of observations confirm that ferritin's role as a biomarker of CMDs is mainly related to the development of T2D but led to more questions owing to dissimilar patterns of association with other CMDs.

Chapter 6 addressed the final research question of this doctoral study by exploring whether ferritin is associated with the risk for cardiovascular complications among people with T2D. Interestingly, ferritin levels were negatively associated with the development of CVD, mainly CHD, in people with T2D in both cross-sectional and prospective studies. Moreover, this association was consistent in two populations that have different cardiovascular risks. Therefore, the pattern of iron status and cardiometabolic risk in people affected by T2D appears to differ from that in the general population, in which a positive association has been more commonly described.

The nature of the inverse ferritin-CVD association in people with T2D requires further research in epidemiological or basic studies to identify potential for survival bias, confounders, mediators, and physiological mechanisms that could contribute to the association.

Table 22 summarizes findings and also characteristics of the studies in this PhD thesis, including data on cut-points for high ferritin in each analysis which will be used later in this discussion.

Table 22 Characteristics and findings of the analyses conducted in each chapter of this PhD thesis.

Chapter	Population/ study	Design	Sample size	Iron markers	Ferritin levels median (µg/L)	High Ferritin cut-point (µg/L)	Main Outcome	Outcome prevalence	Outcome incidence	Exposures and outcomes Association found ("Significant" refers to P<0.05 after adjustments)
2	Spanish children	Cross-sectional/ Prospective	725/ 179	Ferritin Transferrin	Girls: 36 Boys: 35.9	66.6th percentile Girls 44.8 Boys 46.0	MetS Score	NA	NA	-Cross-sectional positive with MetS (Not significant) -Significant decrease in change in MetS score over follow-up by increasing SD units of ferritin **Beta (95%CI) -0.02 (-0.05 to 0.004) P= 0.099**
2	Chilean children	Cross-sectional/ Prospective	Total 567 (16-17 years old) 270 girls 297 boys	Ferritin	Girls: 19.1 Boys: 32.7	66.6th percentile Girls 24.2 Boys 40.9	MetS Score	NA	NA	-Significant (boys) Cross-sectional positive **Beta (95%CI) 0.21(0.06 to 0.37) P < 0.05** Longitudinal positive **Beta (95%CI) 0.23(0.04 to 0.42) P < 0.05** - Pattern of sustained higher ferritin across childhood and higher MetS score (boys) **Beta(95%CI) = 0.31(0.11 to 0.52)**
3	Adults/ SHeS 95-98 (Scotland)	Cross-sectional	Total 8653 2907 Pre-MW 1739 Post-MW 4007 Men	Ferritin	Pre-MW: 31 Post-MW: 59 Men: 96	75th percentile Pre-MW: 48 Post-MW: 93 Men: 152	High BP High WC Low HDL-C	Pre-MW: High BP 20.2% High WC 35.3% Low HDL-C 21. % Post-MW:	NA	-Significant association only with high WC in men and low HDL-C in Post-MW

Chapter	Population/ study	Design	Sample size	Iron markers	Ferritin levels median (µg/L)	High Ferritin cut-point (µg/L)	Main Outcome	Outcome prevalence	Outcome incidence	Exposures and outcomes Association found ("Significant" refers to P<0.05 after adjustments)
								High BP 55.8% High WC 57.1% Low HDL-C 23.6% Men: High BP 51.1% High WC 37.6% Low HDL-C 14.7%		
3	Adults /VIKING (Scotland)	Cross-sectional	Total 2047 589 Pre-MW 625 Post-MW 833 Men	Ferritin	Pre-MW: 25.5 Post-MW: 65.9 Men: 141.3	75th percentile Pre-MW: 42.9 Post-MW: 102.2 Men: 233.5	MetS	Pre-MW: 8.7% Post-MW: 22.1% Men: 22.2%	NA	-Not significant associations ferritin-MetS **Pre-MW** **OR(95% CI) 1.02 (0.42-2.46)** **Post-MW** **OR(95% CI) 1.09 (0.62-1.90)** **Men** **OR(95% CI) 1.43 (0.83-2.46)**
4	Adults/ Croatia-Vis (Croatia)	Cross-sectional	Total 725 151 Pre-MW 290 Post-MW	Ferritin sTfR	Pre-MW: 25 Post-MW: 55 Men: 90	75th percentile Pre-MW: 41 Post-MW: 84 Men: 138	MetS	Pre-MW: 51% Post-MW: 88.6% Men: 50.7%	NA	-Significant positive association ferritin-MetS **Post-MW** **OR(95% CI) 1.71 (1.12-2.62)**

Chapter	Population/ study	Design	Sample size	Iron markers	Ferritin levels median (µg/L)	High Ferritin cut-point (µg/L)	Main Outcome	Outcome prevalence	Outcome incidence	Exposures and outcomes Association found ("Significant" refers to P<0.05 after adjustments)
			284 Men							**Men** **OR(95% CI) 1.78 (1.31-2.42**) -Not significant association sTfR-MetS
5	Adults/ SHeS 95-98 (Scotland)	Prospective	Total 6497 2239 Pre-MW 1343 Post-MW 2915 Men	Ferritin	Pre-MW: 31 Post-MW: 59 Men: 97	75th percentile Pre-MW: 47 Post-MW: 92 Men: 152	Diabetes CHD CEVD	NA	Diabetes 4.9% CHD 5.3% CEVD 2.4%	-Significant positive association ferritin-diabetes **HR(95% CI) 1.59 (1.10-2.34)** - with CEVD not Significant by using quartiles but significant by comparing sextiles **HR (95% CI) 2.08 (1.09–3.94)** - Not significant association ferritin-CHD **HR (95% CI) 1.07 (0.76-1.51)**
6	Adults with T2D / ET2DS (Scotland)	Cross-sectional/ Prospective	Total 821 399 women 422 men	Ferritin	Women: 56 Men: 96.5	80th percentile Women: 120 Men: 199	CHD CEVD	CHD 30.5% CEVD 8.6%	CHD 7.1% CEVD 4.7%	Significant with CHD Cross-sectional HR (95% CI) 0.81(0.67-0.97)* Longitudinal (including recurrent events) **HR (95% CI) 0.39(0.16-0.94)** Longitudinal (excluding

Chapter	Population/ study	Design	Sample size	Iron markers	Ferritin levels median (µg/L)	High Ferritin cut-point (µg/L)	Main Outcome	Outcome prevalence	Outcome incidence	Exposures and outcomes Association found ("Significant" refers to P<0.05 after adjustments)
										prevalent cardiovascular events) **HR (95% CI) 0.28 (0.07–1.14)** *(P=0.078)*
6	Adults with T2D / SIDIAP(Spain)	Cross-sectional	Total 38617 19487 women 14757 men	Ferritin	Women: 46.1 Men: 97	80th percentile Women: 121.1 Men: 244.1	CHD CEVD	CHD 15.9% CEVD 7.3%	NA	-Significant inverse association with CHD **HR (95% CI) 0.62 (0.56-0.69)** and CEVD **HR (95% CI) 0.61 (0.55-0.66)**
NA, Not apply. OR: Odds ratio. HR, hazard ratio. Pre-MW, premenopausal women. Post-MW, postmenopausal women.										

7.2 Findings in the context of criteria for causality

The aim of this doctoral thesis was to describe associations between iron markers and cardiometabolic risk factors and outcomes rather than making conclusions about causality. However, contextualising my findings and reviewed literature against the different causality criteria (temporal relationship, strength of association, biological plausibility, reversibility, consistency, specificity, coherence, and analogy) postulated by Bradford and Hill (236), is useful in the discussion of the role of iron parameters as biomarkers of cardiometabolic risk:

I conducted several longitudinal analyses that confirmed a temporal relationship between ferritin levels and cardiometabolic outcomes. However, both low and high ferritin levels were associated with cardiometabolic risk, suggesting that other factors influence the association. For example, the association between low iron status and CMD risk may only be observed in certain populations such as children or people affected by T2D. As I mentioned previously, the goal of this thesis was not to attempt to identify iron as an aetiological factor, but rather to explore the association between markers of iron status with different measures of cardiometabolic risk both before and after adjusting for key co-variates. A very recent review pointed out common misconceptions in the way prediction and aetiology have been presented in past and current literature (237). In prediction, confounding is not an issue because understanding disease is not its aim but obtaining an explanatory model composed of a strong combination of disease predictors (237). According to this review, absolute risk must be used in prediction, and relative risk in aetiological studies. Unlike aetiological studies, in prediction research, adjustment for confounding variables and causal interpretation should not be conducted (237). My PhD work appears to meet the criteria for classification as an aetiological study. However, I examined consistency of the association patterns in diverse populations to examine generalisability and considered that iron might not be a causal factor in the development of CMD, which overlaps a little with the prediction approach.

In most of the analyses, a dose-response relationship with cardiometabolic risk was also observed in terms of increasing association across quantiles of ferritin distribution, and a positive linear association between serum ferritin as continuous

variable and CMD risk. The linear relationships with serum ferritin were more evident for the outcomes of MetS Z score (Croatia-Vis) and T2D in adults analysed in this thesis, than for MetS and CVD in general adult populations. The adjusted relationships of serum ferritin with incident CEVD in SHeS (positive association) and with incident CHD in people with T2D from the ET2DS (negative association), were identified from the difference between the lowest and highest values of ferritin distribution and a dose response relationship was not always observed. However, as stated by Bradford and Hill (1965), the absence of a dose-response relationship does not exclude causal relationships (236).

The strength of the association between serum ferritin and CMDs was in general weak to moderate. This was expected since CMD is multi-factorial and usually develops as a consequence of interaction between lifestyle, nutritional, environmental, and genetic factors. Therefore, serum ferritin, as for many potential biomarkers, is only likely to make a partial contribution to the risk. Although a moderate association does not rule out a biomarker as a potential causal factor (236), in the case of ferritin, this feature tilts the balance towards alternative mechanisms or explanations behind the relationship between iron metabolism and cardiometabolic risk. The discussion of this latter aspect links to the next causality criterion: biological plausibility. Iron is involved in biochemical reactions which generate free radicals (superoxide and hydroxyl ions) that have a potentially harmful effect on insulin signalling and endothelial function (11). Increased oxidative stress derived from body iron excess is the main theoretical underlying mechanism proposed to date for linking iron and cardiometabolic risk. IR observed in *in vitro* research with adipose, hepatic, and endothelial cells exposed to iron overload environments, provides evidence of iron causing CMD-related metabolic alterations (238-241). However, there are certain issues to be considered here. First, there is potential for reverse causality because increased oxidative stress can increase iron proteins in tissues and thus their circulating levels (225, 242-244). This might be a defensive counter-mechanism to avoid generation of free radicals because iron proteins such as ferritin and transferrin catch circulating free ferrous iron and prevent its use in redox reactions. Second, there is no epidemiological evidence on oxidative stress acting as a link between ferritin and cardiometabolic risk. To date, there are no longitudinal

studies on iron markers and MetS or T2D that have adjusted the associations for markers of oxidative stress to test possible mediating or co-incident effects. Similarly, some cross-sectional studies have reported higher levels of ferritin and oxidative stress in subjects with MetS, but in these studies, the oxidative stress markers were not used as covariates (146, 149).

Reversibility is a key point in establishing causality, and research on iron depletion and cardiometabolic risk has shown discrepant findings. Frequent blood donations have been associated with a reduction of IR and concomitant iron depletion in cross-sectional studies (245). In contrast, another cross-sectional study and a prospective study of 33,541 individuals did not find associations of blood donation with insulin sensitivity and development of T2D, respectively (246, 247). However, phlebotomy or blood donation has effects in addition to decreasing body iron stores. Frequent blood donation reduces risk of clotting and thrombosis presumably by replacement of old blood products by new ones (248), and this can also be expected to reduce risk of myocardial infarction and stroke. Frequent blood donors are also be more likely to be more health conscious than people who do not donate blood (249).

The consistency of the association between iron and cardiometabolic risk varies depending on the outcome. While the ferritin-T2D association has been highly consistent across several studies as well as in this doctoral study, the ferritin-MetS association is very heterogeneous despite the significant pooled association reported in the meta-analysis presented in Chapter 3 of this thesis. Furthermore, all the studies included in the meta-analysis reported a significant association in at least one of the sex/menopausal status groups, but I identified discrepancies in the significance of the association in these groups in several studies.

It is also noteworthy that serum ferritin was not independently related to MetS in the cross-sectional VIKING study but was independently associated with incident T2D in the SHeS 95–98 prospective follow-up study. If a biomarker is associated with CMD with apparently no marked confounding effects from other CMD risk factors, it would be expected to show the same pattern with a cluster of risk factors such as MetS. In other words, if MetS is on the causal pathway from ferritin to incident T2D,

BMI, the main confounder of the ferritin-MetS association in the VIKING study, could have been expected to significantly attenuate the association with the development of T2D in the SHeS 95–98. BMI was slightly higher in the VIKING than in the SHeS 95–98 longitudinal study [median (IQR) 26.7(24.1–29.9) vs. 25.7(23.1–28.6) kg/m^2]. Although this difference might not be strong enough to support the discrepancy between the studies, it is possible that the BMI-ferritin relationship differs between populations, even those from within the same geographical region, in this case Scotland. In fact, the VIKING study involved inhabitants of Shetland Islands who are in genetic terms "isolates". There are also differences in distribution of obesity, physical activity levels and diabetes prevalence between Shetland and Scotland as a whole. Prevalence of obesity is higher in Shetland than in Scotland (32 % v. 27.4%) (250), but physical activity levels and prevalence of diabetes are respectively higher and lower in Shetland than Scotland (recommended levels of physical activity 41% v. 38%; diabetes 4.7% v. 5.3%) (250, 251). Another explanation for the contradictory findings can be that increased iron stores could lead to T2D via pathways other than the intermediate onset of MetS or its components, which are closely related to IR. This explanation is consistent with the finding from several studies that the ferritin-T2D association remains significant despite adjusting for variables related to MetS and IR, such as HDL-C level, blood pressure, and WC. Moreover, two studies involving Korean men reported an association between serum ferritin and T2D, independently of the effect of the IR index (HOMA-IR) at baseline and several covariates (252, 253). Despite the above hypotheses, it is also important to consider the greater potential for residual confounding in the prospective SHeS 95-98 study than in the cross-sectional VIKING, since there may have been variations in the BMI during the follow-up.

The assessment of consistency in the relationship between serum ferritin and CEVD still requires further research since my review of the literature using relevant keywords identified only two longitudinal studies (196, 197).

Specificity, coherence, and analogy are the remaining causality criteria to discuss. Currently, specificity, defined as the increased risk by an exposure variable for a particular outcome and no other outcomes, is uncommon given the complex

interactions between several risk factors for non-communicable chronic diseases. Iron excess has been related to cancer as well, under similar likely underlying mechanisms mentioned previously (254). The proposed relationship and underlying mechanism between iron and cardiometabolic risk are coherent with the development of CMD in terms of metabolic alterations derived from the pro-oxidant properties of iron. With regard the criterion of analogy, as with iron, antioxidant vitamin levels have also been evaluated as biomarkers of CMD risk on the basis of the hypothesis of the involvement of oxidative stress in the aetiology of these diseases. Previous large cohort studies have reported inverse associations between serum antioxidant vitamin concentrations such as vitamins C, E (tocopherol levels), and A (retinol levels) and development of CMD (255-257). However, supplementation with antioxidant vitamins has not shown an effect on prevention of CMD (258). Research on dietary intake of vitamin E intake as a protective factor for incident T2D has shown inconclusive results (259, 260).

Increased iron stores, estimated as serum ferritin levels, appear to meet many of the Bradford –Hill criteria to be considered as a potential etiological factor for T2D, but with conflicting findings with respect to other CMDs. Combining the results of my doctoral research with existing literature, I agree with Jehn et al's. (2007) comment that increased ferritin might be either a causal factor and/or one of the several metabolic abnormalities in the course to T2D (see Chapter 5 for a detailed discussion of the study and the limited power contributing to the failure of the study to determine a statistically significant independent association) (20). In my opinion, this flexible and more realistic conclusion could be extended to the relationships of serum ferritin with MetS, CHD, and CEVD as well as to the association with T2D. In addition, I found that low iron status appears to be associated with increased risk of CMD in some populations, but this pattern of association is observed much less frequently than with iron excess. The underlying mechanism behind this inverse associations remains to be explored.

7.3 Conflicting findings among iron markers

The directions of associations between different iron markers and variables of cardiometabolic risk were inconsistent. This observation provides a strong argument

against the notion of iron excess as cardiometabolic risk factor. If iron excess is involved in cardiometabolic risk, one would expect different iron markers to show complementary patterns of association with CMD. In line with my findings, Podmore et al. recently described positive associations between ferritin and transferrin and incident T2D and an inverse association between TSAT and incident T2D in the EPIC-InterAct study (195). A positive association with TSAT would be expected if iron excess were a risk factor for T2D. Podmore et al. suggested that inflammation may influence the association with TSAT, and that IR may lie on the causal pathway between ferritin or transferrin levels and T2D. This hypothesis is not supported by the finding that the ferritin-T2D relationship appears to be independent of the IR index (252, 253) as mentioned previously, although there are no studies on transferrin and T2D with similar adjustment. Podmore et al. also speculated a possible protective effect of TSAT on T2D risk, as higher TSAT indicates better scavenging of free circulating iron (195). These discordant association patterns show that iron proteins are accounting for something beyond iron metabolism in relation to cardiometabolic risk. This idea is even more plausible if we consider that although serum ferritin was inversely correlated with transferrin and sTfR as expected, this correlation was weak, not reaching more than 0.2–0.4, as described in Chapters 2 and 4.

7.4 Reliability of serum iron biomarkers as a measurement of iron status

The majority of the analyses throughout this thesis have shown that serum ferritin, transferrin, and sTfR are associated with cardiometabolic risk or IR in unadjusted or adjusted analyses. Serum iron biomarkers might reflect physiological processes other than iron metabolism, as discussed above. If serum iron biomarkers are altered owing to underlying mechanisms in the sub-clinical phase of CMD, their concentrations may not entirely reflect tissue levels to assess iron deficiency in individuals with higher cardiometabolic risk or IR. The effect of hepatic disease or acute inflammation on ferritin levels is well known, and the role of these factors should always be considered when high ferritin levels are observed (68). However, serum ferritin could show a subtle increase and still remain within the normal range in individuals with higher cardiometabolic risk or IR, masking an iron deficient status.

Conversely, higher sTfR levels may not indicate true iron overload if hyperinsulinemia and not iron homeostasis increases the synthesis of this protein, as suggested in Chapter 4. Admittedly, testing the above hypothesis is difficult since the gold standard of iron measurement is iron present in bone marrow tissue (261).

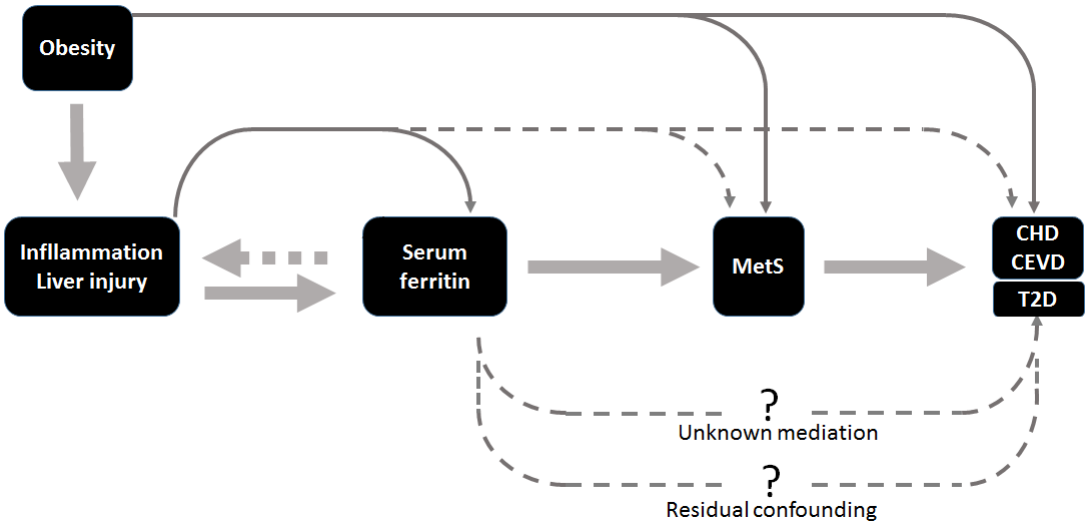
7.5 Complexity of the iron biomarkers-cardiometabolic risk relationship

Identifying biomarkers of cardiometabolic risk requires understanding of the complex interaction of many factors and it is challenging to reach a conclusion regarding causality. In my opinion, there are three main axes of influence on the iron-cardiometabolic risk association: adiposity, hepatic function, and inflammation. Figure 11 depicts the complex role of potential confounding and mediation by obesity (excess adiposity), inflammation and liver injury in the association between markers of iron status and cardiometabolic risk. As mentioned in the introductory chapter, excess adiposity can be considered a confounding factor since overweight or obesity increase the risk of MetS and CMD and at the same time, a person who is overweight may have higher ferritin owing to a higher number of adipocytes with stored iron. Overweight/obesity is unlikely to be a mediating factor between ferritin and CMD because so far and to my knowledge, there is no evidence that increased ferritin or iron stores directly or indirectly lead to increased adipogenesis or food intake. Inflammation is also a confounding factor since it has been associated with T2D and cardiovascular disease, and with higher serum ferritin because ferritin is an acute phase reactant as well. However, theoretically, inflammation may also be a mediating factor or intermediate variable if iron-induced oxidative stress triggered inflammatory pathways (262). This same circular relationship may occur with hepatic injury. Liver damage may lead to metabolic alterations and subsequent cardiometabolic risk along with the release of ferritin molecules from damaged hepatocytes to the bloodstream reflecting an apparent association between ferritin and cardiometabolic risk. On the other hand, if increased hepatic iron generates liver damage via oxidative stress (263), liver injury would be a meditating process between iron status and CMD. Overweight/obesity can promote subclinical inflammation and liver injury (as non-alcoholic fatty liver disease), so this covariate may be considered a predecessor of the confounding or mediating effects of

inflammation and liver injury on the ferritin-cardiometabolic risk association. Despite these interrelationships that may imply collinearity, I did not identify changes in effect estimates that suggested the presence of collinearity by using concomitant adjustments for BMI, CRP, and transaminase levels in the same model. However, formal collinearity tests were not conducted. In addition, adjusting for covariates that are intermediate variables may lead to over-adjustment and estimates of the effect that are biased towards the null (264). On the other hand, this kind of adjustment allows investigation of direct effects (264). The adjustments conducted across the studies in this thesis showed attenuating effects of transaminases as markers of liver injury and BMI, but not of inflammatory markers, on the association between higher serum ferritin and cardiometabolic risk variables. However, in at least half of the analyses in this thesis, the statistical significance of the association persisted. Therefore, liver injury and excess adiposity partially explain the ferritin-cardiometabolic risk association, and there might be residual confounding or mediating effects owing to the limitations in the measurements of the covariates used or unknown factors still to be disclosed. These unknown factors are a key target to test alternative pathways from altered serum ferritin to development of T2D that differ from the intermediate phase of MetS. As described before, this is highly likely given that adjustments for MetS components do not abolish the statistical significance of the ferritin-T2D association. On the other hand, the association between ferritin and CHD appears to be confounded or mediated by adiposity and cardiovascular risk factors since the adjustment models with these covariates attenuate the association. Ferritin-CEVD relationship still requires further research for clearer conclusions.

In terms of the data available, with the covariates of adiposity, inflammation, hepatic injury, and the rest of adjustment variables I attempted to cover a relevant set of factors of potential concomitant influence on exposure and outcome variables, to limit confounding (265).

Figure 11 Diagram illustrating potential complex roles of confounding/mediating factors in the ferritin-cardiometabolic risk relationship

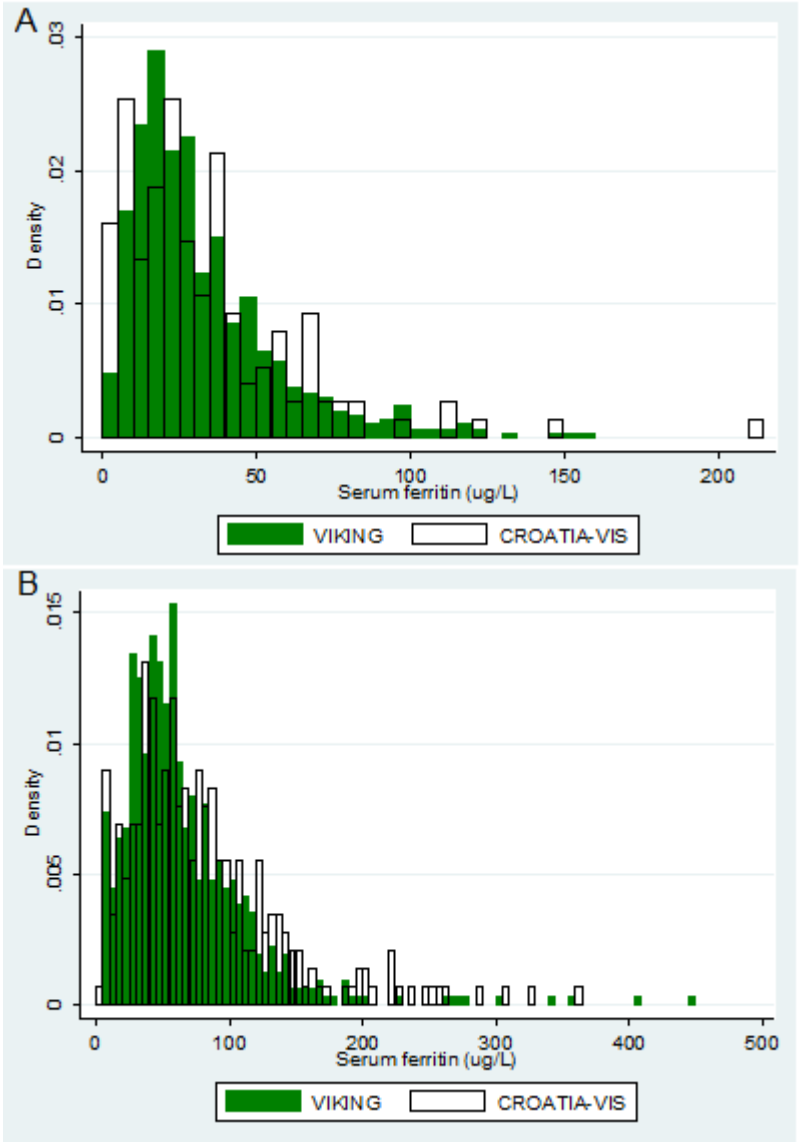


7.6 Distribution of ferritin levels across study populations

When compiling all the findings from each chapter of this thesis, it is important to compare the distribution of ferritin between the populations studied to consider whether these might contribute to the findings. Figures 12 and 13 show histograms of ferritin levels in women and men from the VIKING and Croatia-Vis study, in which I identified non-significant and significant associations of ferritin with MetS, respectively. Premenopausal and postmenopausal women from both studies had similar ferritin distributions (Figure 12), men from the VIKING study had a slightly more skewed distribution towards lower ferritin values than men in the Croatia-Vis study (Figure 13). The higher ferritin levels in the Croatia-Vis study may have influenced the significant association with MetS found, but as commented previously, the lack of adjustment for hepatic injury markers may also have contributed to a stronger association in the Croatia-Vis population than in the Shetland population. The distribution of ferritin levels was very similar in the Scottish population of VIKING and SHeS prospective study (Figure 14). Thus, differences in ferritin distribution do not seem to contribute to the lack of association between ferritin and MetS in VIKING and the positive association with incident T2D in the SHeS 95-98. I also compared the ferritin distribution in the Scottish population

of patients with T2D in the ET2DS and general population of the SHeS 95-98, since these studies showed opposite patterns in the direction of the associations between ferritin and CVD (Figure 15). I used an age range of 60-75 years in the SHeS for comparability with the ET2DS. In both postmenopausal women and men, there was a slightly higher density of cases in lower values of ferritin (<30 μg/L approximately) in the ET2DS than in the SHeS. However, the ferritin distribution in the highest levels was similar in both studies (Figure 15). In fact, cut-points for high ferritin (highest quintile) in the ET2DS and the SHeS were comparable [postmenopausal women: ET2DS 120 μg/L, SHeS 111 μg/L; men: ET2DS 198 μg/L, SHeS 183 μg/L]. Conversely, cut-points for low ferritin (lowest quintile) were slightly lower and much lower in postmenopausal women and men, respectively, of the ET2DS than in the SHeS study [postmenopausal women: ET2DS 21 μg/L, SHeS 31 μg/L; men: ET2DS 36 μg/L, SHeS 60 μg/L]. The above observations suggest that the inverse association between ferritin levels and CVD in people with T2D does not appear to be explained by major differences in ferritin distribution in respect to a general population of adults.

Figure 12 Distributions of serum ferritin in women of the VIKING (Chapter 3) and Croatia-Vis (Chapter 4) studies



A, Premenopausal women. B, Postmenopausal women.

Figure 13 Distributions of serum ferritin in men of the VIKING and Croatia-Vis studies

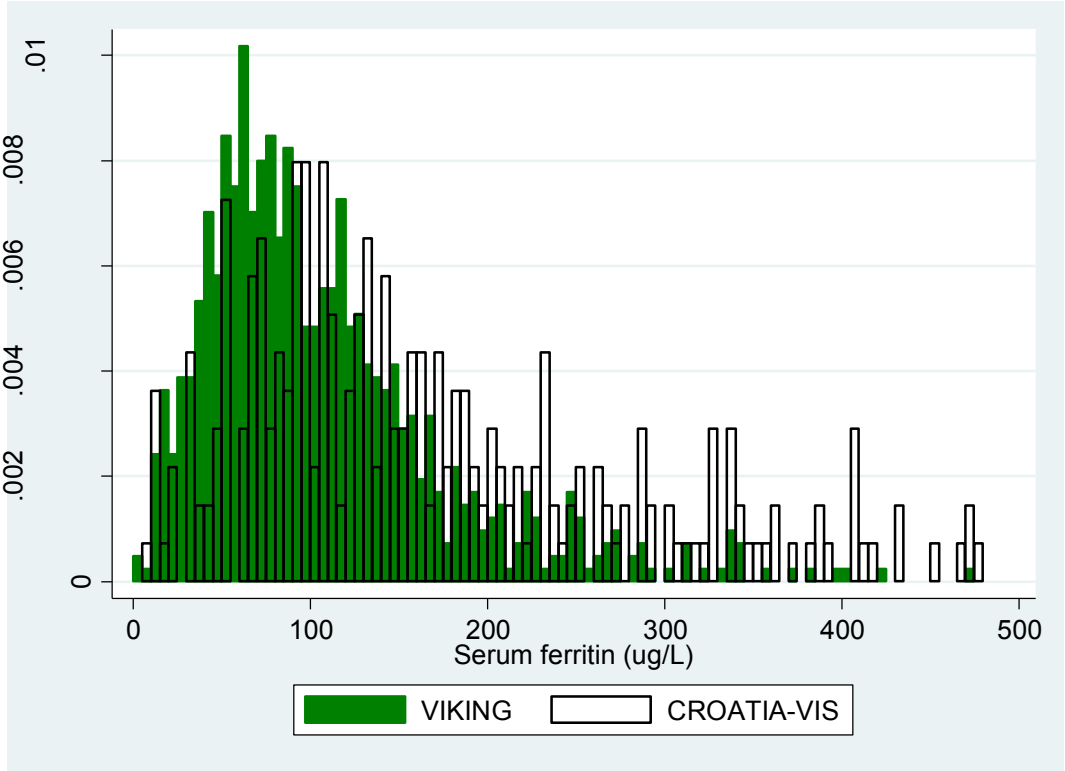
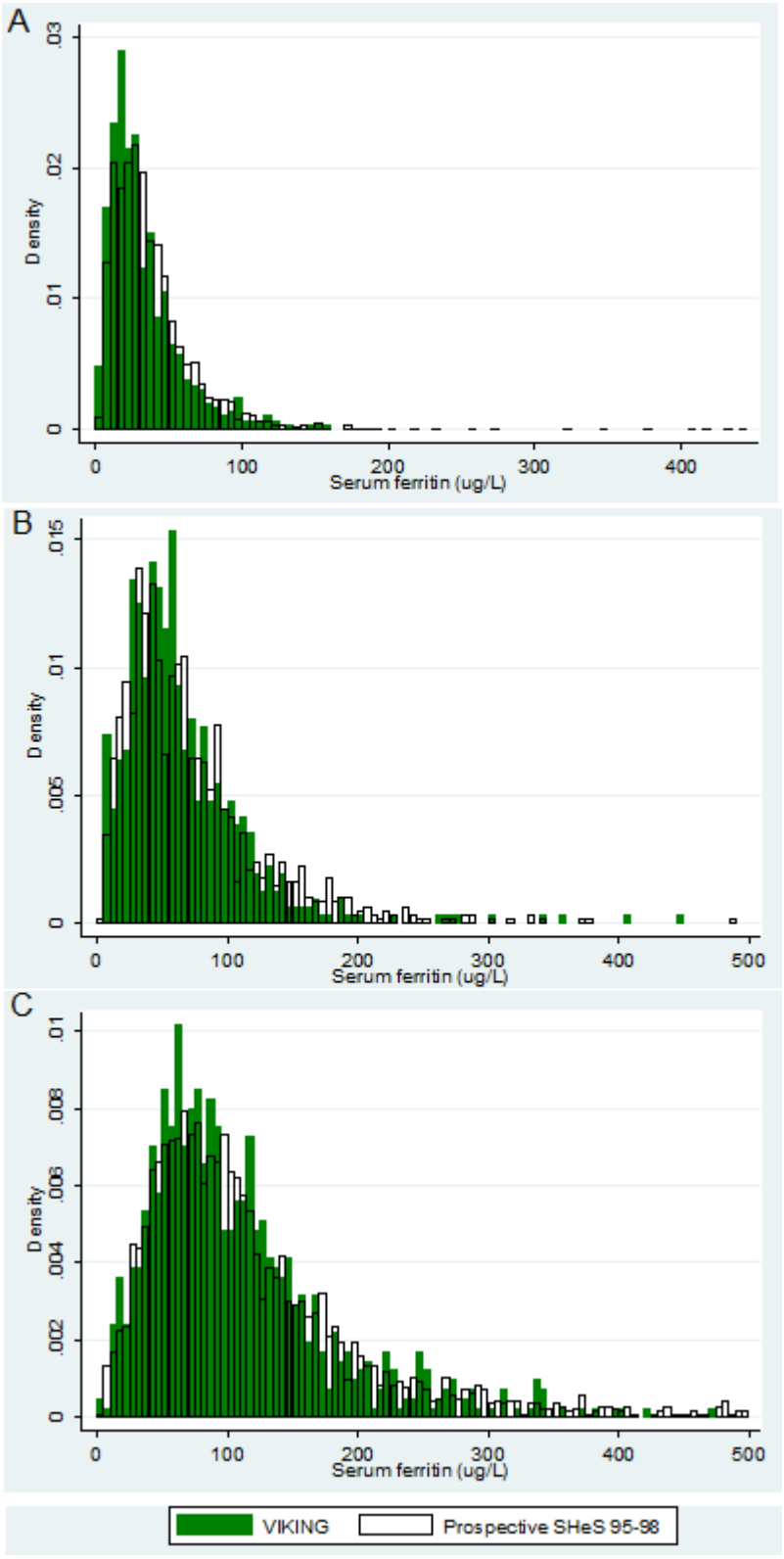


Figure 14 Distributions of serum ferritin in women and men of the VIKING and SHeS 95-98 (Prospective study)



A, Premenopausal women. B, Postmenopausal women. C, Men.

Figure 15 Distributions of serum ferritin in subjects of the Scottish studies in people affected by T2D and in general population of 60-75 years of agewomen. B, Men.

7.7 Strengths of the research

This PhD thesis has made a unique contribution to the current knowledge base through the exploration of association patterns of serum ferritin and other iron markers with cardiometabolic risk in populations of children, adults, and people with T2D, by conducting several secondary data analyses. Different stages of cardiometabolic risk were investigated with both MetS, a key risk factor for CMD, and CMD outcomes, both considered depending on data availability.

As a result of my collaborations with research groups leading projects in different countries, I was able to study the associations in datasets from populations of different geographical origins. These collaborations also allowed me to provide information on ferritin and cardiometabolic risk in Latin-American, Scottish, and Croatian populations for which there were few or no previous studies on the association between ferritin and cardiometabolic risk. Consequently, it was possible to investigate the consistency of the association across different populations with different distributions of iron markers and different baseline levels of CMD while taking into account differences in design, power, and the different variables available in the datasets.

This PhD thesis adds new information on the association of iron markers and MetS in children, something that had not been explored in previous studies. The evaluation of the association of sustained patterns of high ferritin levels across childhood and

MetS in adolescence is another novel contribution of this doctoral thesis to the current literature on iron and cardiometabolic risk. This approach, using repeated measurements of ferritin was previously recommended by Jehn et al. (2007) to characterise the consistency of the association with T2D over time, and this applies to other CMDs as well. Cohort studies of cardiometabolic risk outcomes do not often have measurements of iron markers at several time points of follow-up. However, I had the opportunity of examining three measurements of ferritin at different time points from childhood to adolescence in the cohort of Chilean children. The association between serum ferritin and MetS appears to be consistent in children/adolescents as opposed to that observed in adults.

The sixth chapter on ferritin and cardiovascular complications in people affected by T2D is another strength of this thesis since this is the first analysis of an association by reporting statistical adjustments, with a longitudinal design and multi-cohort approach.

With the exception of the prospective analysis involving 170 Spanish children described in Chapter 2, the different studies in this doctoral study included relatively large samples. This observation also applies, to a less extent, to specific demographic groups I evaluated, such as children and people affected by T2D, for which very large datasets are not as common as for the general populations. Nevertheless, I was fortunate to work with a very large sample of people with T2D – the SIDIAP study. Most of the analyses included study samples of around 70–97 % of the original samples, except the analyses on ferritin and CMD in the SHeS 95–98 (37.6%) and on ferritin and CVD in people with T2D from the SIDIAP study (12.1%). Only in this latter study there was a considerable chance for selection bias since ferritin measurements were conducted in individuals who were suspected as having iron deficiency or anaemia. However, given that the associations in this study were similar to those found in the subjects affected by T2D from the ET2DS, it appears unlikely that the associations of the SIDIAP study have been affected by selection bias. Similarly, there were some differences between excluded and included subjects in the remaining analyses, which might indicate that the samples selected were not representative of the original populations. It is difficult to estimate to what extent

these differences may have influenced the findings, if that was the case. Since the criteria for selection consisted of having data available for outcome/exposure variables and covariates, this means that the valid cases in multivariable models would correspond at any rate to the samples selected. It is important to take into account that differences between included and excluded subjects are not always consistent, as described in Chapter 5, and may also be chance findings.

7.8 Limitations of the research

There are, however, various limitations to the analyses in this thesis. Although in general, the set of covariates was homogeneous across the different analyses in the chapters, there were some analyses in which hepatic injury markers were not available, so the magnitude of the independent association between serum ferritin and CMDs may be overestimated. Similarly, the estimation of ORs leads to overestimation of the risk ratio when the outcomes are common (around > 10%)(266). There might have been overestimation of the cross-sectional associations in adults between the iron markers and MetS, since MetS prevalence was 18% in the VIKING study and higher than 50% in postmenopausal women and men from the Croatia-Vis study. This is not relevant to the cross-sectional analyses for associations between the iron markers and specific MetS components with low prevalence. However, overestimation of cross-sectional associations is not a major concern given that most of the associations described in the chapters 3 and 4 were not statistically significant and so the conclusions drawn are appropriate.

The use of different populations in the various analyses was previously mentioned as a strength of this PhD thesis. However, it would have also been interesting to evaluate the consistency of the association of ferritin from early risk to development of CMD in the same population, since the heterogeneity from different data sources hinders us from arriving at more solid conclusions.

I worked with available measurements of transferrin and sTfR as additional iron markers with respect to MetS in Spanish children and Croatian adults, respectively. Unfortunately, the data for iron markers other than ferritin were limited. In fact, at the beginning of my PhD, along with my supervisors, I looked for collaborations

with research groups in Cambridge and Potsdam which are part of the EPIC study, to accomplish a study with multiple iron biomarkers. However, we obtained negative answers since these groups already had plans for their own analyses. The recent EPIC-Interact study on multiple iron markers and T2D by Podmore et al. is an example of a PhD thesis conducted with the information I tried to work with. Investigations of the association between multiple iron markers with the outcomes of MetS, CHD, and CEVD are required in other large and robustly-adjusted multi-cohort studies.

While examining repeated measurements of ferritin across childhood and their relationship with MetS at adolescence is described as one of the strengths of this doctoral thesis, this kind of evaluation is also urgently needed in the adult population, in whom the link between iron and CMD has been more widely investigated. I made an effort to include this approach in my doctoral study after collaborating with the research group in charge of the Kuopio Ischemic Heart Disease Risk Factor Study (KIHD). This may be the only prospective cohort study with repeated measurements of serum ferritin (0, 4, 11, and 20 years' follow-up) and outcomes of T2D, acute myocardial infarction, and stroke. However, there were only 725 men free of T2D at baseline for whom complete data were available. The limited statistical power meant that no significant association between ferritin levels at each time point and incident T2D during 11 or 20 years' follow-up was identified after adjustment for covariates. The slope for change in ferritin level per year was not associated with T2D either. Since the basic association between ferritin at single time points and incident T2D was not successfully replicated in a cohort which has reported a significant association in larger samples (27), the repeated measurement analysis did not make sense. The findings from this exploration would have been inconclusive owing to type II error. Therefore, whether trajectories of serum ferritin in adults are associated with onset of MetS, T2D, or other CMD, should be explored in further research that will require specific grants to fund repeated measurements of iron markers in serum banks of large cohort studies. Along with my supervisors I applied for a grant from the Chief Scientist Office of Scotland in 2013 to fund repeated measurement of ferritin and hepcidin in serum samples across the follow-up of the Whitehall II study, but the application was unsuccessful.

Regardless of high statistical power and the longitudinal and/or independent nature of some associations, it is important to bear in mind that the findings described in this thesis are preliminary, in terms of making policy decisions about the value of iron markers for assessing cardiovascular disease risk.

7.9 Future research

This doctoral research addressed several questions, which in turn generated additional questions. There still are many gaps in the understanding of the iron-cardiometabolic risk link that need further evaluation and which were not studied in this PhD research owing to reasons of space, time, and feasibility. The characterisation of the ferritin-cardiometabolic risk association needs additional observational studies and meta-analyses, mainly with regard to specific populations such as children and people affected by T2D. Mendelian randomisation and metabolomics are analytical tools that might provide new and better-quality evidence on the underlying mechanisms and/or mediating factors and I discuss these in more detail below.

The association patterns found in paediatric populations require confirmation in other studies with pre-pubertal children and adolescents, with research designed to provide a clearer understanding of the biological mechanisms or the role of residual confounding. Studies in pre-pubertal children with narrower or more specific age ranges would clarify the coexistence of inverse and positive associations between ferritin and cardiometabolic risk factors observed in the Spanish cohort described in Chapter 2. As mentioned previously, whether patterns of the iron markers over time are related to risk for CMD in adults remains to be studied, but having found it to be related to MetS during childhood is a relevant insight for future research.

The associations reported in children and people affected by T2D require more studies and subsequent systematic reviews/meta-analyses to provide more robust evidence about whether iron markers make a useful contribution to cardiometabolic risk. In particular, the association between serum ferritin and CEVD requires further large studies with a large number of participants given the ambiguity in the significance of this relationship in the Scottish cohort described in the thesis.

The adjustment models used throughout this PhD thesis were predetermined under the assumption of potential modifications or confounding effects on the associations evaluated, and the use of the covariates has been justified in the introductory chapter. However, it is important to conduct large longitudinal analyses looking for independent predictors of serum ferritin and other iron makers among a wide range of variables (nutritional, inflammatory, adipokines, lifestyle). These studies, which might be based on secondary data analyses, would help characterise the set of covariates and understand the role of circulating iron proteins in other possible physiological contexts. I noticed that the vast majority of the articles reviewed for this thesis did not provide any rationale for the covariates they used.

As previously discussed, the involvement of different unknown pathways linking increased ferritin and cardiometabolic risk is highly likely. Therefore, the use of novel approaches to explore mediating processes or new metabolic pathways is extremely pertinent.

One of these approaches is "metabolomics" which consists of the evaluation of the whole set of metabolites in a determined biological system (cell, tissue, organ, biological fluid, or organism), also known as "metabolome" (267). Metabolomics also allows researchers to compare the metabolites (low molecular weight molecules) in terms of their quantity or absence/presence according to given characteristic such as disease or exposure to a specific factor (267, 268). This approach takes advantages of large-scale measurements and identification technologies such as mass spectrometry and nuclear magnetic resonance to characterise metabolomes (268). Future research could focus on characterisation of metabolomes in baseline serum samples of individuals with high (not biased by inflammation) and normal ferritin levels, who have developed MetS or T2D during a follow-up in comparison with controls without these outcomes. With the metabolite patterns obtained from such measurements, it would be possible to conduct subsequent analyses to identify metabolic pathways that link iron biomarkers and development of the CMDs. A similar approach among people with low iron status instead of iron excess and cardiovascular diseases as outcomes in people with T2D may clarify the mediating factors for the association reported in Chapter 6.

In October 2016, I wrote a research proposal on metabolomics for ferritin and T2D in a sub-sample of the KIHD cohort as part of an application for the position of "Professor" at a Colombian university. Coincidentally, a few days later, a publication on this topic was released with MetS as the outcome but using a cross-sectional design plus a clinical trial arm with phlebotomy as the intervention to also characterise changes in the serum metabolome after iron depletion (269). The authors identified higher levels of sarcosine, methionine sulfoxide, and phosphatidylcholines and lower levels of citrulline in individuals with MetS and iron overload (n = 56) than in subjects with MetS but not iron overload (n = 54) and healthy lean controls (n = 53). They hypothesised a link between increased iron and sarcosine, an intermediate of glycine biosynthesis and degradation, which has recently been related to regulation of inflammation and lipid metabolism (269). The pattern of citrulline was described as possibly spurious since it appears that citrulline, an intermediate product in the urea cycle, is an indicator of erythrocyte mass, so it has a positive association with iron status. The authors failed to find beneficial effects after intervention with phlebotomy (until serum ferritin fell to 50–100 μg/L) on the HOMA index, fasting glucose, and lipid profile. However, the study reported higher circulating methionine and lower glutamate after the intervention (269). Longitudinal metabolomic studies with T2D as the outcome are required to establish whether reverse causality may have biased the cross-sectional findings on associations between ferritin and MetS in the above metabolomic study.

Genetic studies can address some of the problems associated with observational studies including reverse causality and confounding when attempting to identify whether iron metabolism is a causal factor or simply a marker of other metabolic processes in the development of T2D and increased cardiometabolic risk. If the key assumptions are fulfilled, Mendelian randomisation studies can provide information about causality (270, 271). The first assumption is the correlation between serum ferritin and different genetic polymorphisms that modify serum ferritin so that the polymorphisms can be used as instruments (270). Second, there are no unmeasured common causes of the polymorphisms and CMDs. Third, every directed pathway from the polymorphisms to the CMDs has to pass through serum ferritin (271). To the best of my knowledge, only one study involving a Chinese population (*n* = 1,574)

used MR to investigate the link between serum ferritin and T2D(272). The authors of this study found that two variants (V736A and D521D) of the gene *TMPSS6*, which encodes the serine protease Matriptase-2 associated with up-regulation of iron absorption via suppression of hepcidin synthesis, were related to lower haemoglobin and serum ferritin and lower risk of T2D in a cross-sectional study(272). They also reported that the association between the *TMPSS6* variants and T2D were attenuated by serum ferritin in a triangulation approach used in the MR study, concluding that ferritin might mediate the associations, and the ferritin-T2D relationship was causal and unconfounded(272). However, the authors acknowledged that the study was preliminary and further research involving larger sample sizes was needed. Future MR studies could target genetic variants related to predictors of ferritin other than *TMPSS6*, possibly including those unrelated to iron metabolism-related proteins to explore alternative pathways or confounders in the link between ferritin and T2D. This approach requires identification of genes that influence ferritin as proposed previously in this discussion.

To conclude and summarise the findings and reflections of this PhD thesis, serum ferritin and other iron markers are associated with cardiometabolic risk, and it is likely that these markers are reflecting something else than iron homeostasis with regard to MetS or T2D, warranting further investigations. Second, the association between iron markers and an intermediate stage of cardiometabolic risk such as MetS, can differ by populations despite a trend of pooled positive association. Third, there could be the coexistence of inverse and positive associations in prepubertal children, and iron deficiency and not iron excess appears to be related to cardiovascular complications in T2D patients. This PhD project has therefore contributed to existing knowledge with additional information regarding the complex relationship between iron metabolism markers, MetS, and CMD. I hope the products of the work will encourage future research to provide deeper understanding of the underlying mechanisms and mediating or confounding factors in this relationship.

7.10 Products of this PhD project

Some findings from this PhD thesis have been disseminated through peer-reviewed publication and poster presentation at academic conferences. The analysis in Chapter

4 was published in the British Journal of Nutrition (BJN) with the title of "Soluble transferrin receptor levels are positively associated with insulin resistance but not with the metabolic syndrome or its individual components" (273). This article does not appear as an appendix since its copyrights belong to BJN and Cambridge University Press. The analyses from Chapter 3 on ferritin and MetS in the VIKING study and SHeS were presented in the 60th Annual Scientific Meeting of the Society of Social Medicine at the University of York in 2016 as a poster presentation (Appendix Abstract Poster presentation published in Journal of Epidemiology and Community Health).

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Appendices

Appendix tables

Table 1. Some definitions for Metabolic syndrome and its components by international organizations

	WHO (1999)	EGIR(1999)	Revised NCEP ATP III (2004)	IDF (2006)	Harmonized definition (2009)
	Glucose intolerance, IGT or diabetes and /or insulin resistance* together with two or more of the following	Insulin resistance (defined as hyperinsulinemia –top 25% of fasting insulin values among the non-diabetic population) plus two of the following	Three or more of the following risk factors	Elevated waist circumference plus other two risk factors	Three or more of the following risk factors
Elevated fasting plasma glucose		≥ 6.1mmol/L(110 mg/dL)	≥ 5.6 mmol/L(100 mg/dL)	≥ 5.6 mmol/L(100 mg/dL)	≥ 5.6 mmol/L(100 mg/dL)
Elevated blood pressure	Systolic ≥ 140 and/or diastolic ≥ 90 mmHg	Systolic ≥ 140 and/or diastolic ≥ 90 mmHg or treatment	Systolic ≥ 130 and/or diastolic ≥ 85 mmHg	Systolic ≥ 130 and/or diastolic ≥ 85 mmHg	Systolic ≥ 130 and/or diastolic ≥ 85 mmHg
Elevated triglycerides	≥ 1.7 mmol/L(150 mg/dL)	≥ 2.0 mmol/L(178 mg/dL) or treatment	≥ 1.7 mmol/L(150 mg/dL)	≥ 1.7 mmol/L(150 mg/dL) or treatment	≥ 1.7 mmol/L(150 mg/dL) or treatment
Reduced HDL-cholesterol	Men: < 0.9 mmol/L(35 mg/dL) Women : <1.0 mmol/L(39 mg/dL)	< 1.0 mmol/L(39 mg/dL) or treatment	Men: < 1.03 mmol/L(40 mg/dL) Women : <1.29 mmol/L(50 mg/dL)	Men: < 1.03 mmol/L(40 mg/dL) Women : <1.29 mmol/L(50 mg/dL) or specific treatment	Men: < 1.03 mmol/L(40 mg/dL) Women : <1.29 mmol/L(50 mg/dL) or specific treatment
Obesity /Elevated waist circumference	Men: waist-hip ratio > 0.90 Women: waist-hip ratio > 0.85 and/or BMI > 30 Kg/m^2	Men: WC ≥ 94 cm Women: WC ≥ 80cm	Men: WC ≥ 102 cm Women: WC ≥ 88 cm	WC ethnicity specific	WC ethnicity specific
Microalbuminuria	Urinary albumin excretion rate ≥ 20 µg/min or albumin:creatinine ratio ≥ 30 mg/g				

WHO, World Health Organization. EGIR, European Group for the Study of Insulin Resistance. NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III. IDF, International Diabetes Federation. IGT, Impaired Glucose Tolerance. BMI, Body Mass Index. WC, Waist Circumference. *Estimated by euglicemic-hyperinsulinemic clamp.

Table 2. Differences between included and excluded children from the Spanish Cohort

	Included (n=726)	Excluded (n=130)	*n* for variables in excluded	P value for difference	P value for difference- Adjustment for age*
Age (years)	8.0(6.5-9.7)	7.8(6.2-8.9)	*n*=130	**0.047**	
Sex % female/male	48.2/51.8	39.9/48	*n*=130	0.553	
Cardio metabolic risk variables					
Waist (cm)	63(53.5-75)	56(52-70)	*n*=122	**0.014**	0.278
SBP (mmHg)	107(99-114)	103(94-109)	*n*=125	**<0.001**	**<0.001**
DBP (mmHg)	60(55-65)	58(53-65)	*n*=124	0.098	
Glucose (mg/dL)	87(83-91)	87(83-90.5)	*n*=109	0.545	
TG (mg/dL)	54(43-74)	55(42-74.5)	*n*=113	0.926	
HDL-C (mg/dL)	55(47-64)	59(50.2-66)	*n*=112	**0.024**	0.086
Insulin (ulU/ml)	4.11(1.51-8.04)	3.04(0.52-7.85)	*n*=62	0.292	
HOMA-IR	0.88(0.31-1.73)	0.84(0.14-1.84)	*n*=57	0.659	
HbA_{1c}(%)	5.3(5.1-5.5)	5.3(5.1-5.5)	*n*=98	0.291	
Iron markers					
Ferritin (µg/L)	36(25-52)	32.8(22.9-45.4)	*n*=108	0.123	
Transferrin (mg/dL)	274(251-298)	265.5(244-293)	*n*=102	0.093	
Covariates			*n*=130		
BMI Z score	0.53(-0.55 to 1.70)	-0.12(-0.75 to 0.83)	*n*=127	**0.001**	**0.004**
CRP (mg/L)	0.7(0.3-2.4)	1.0(0.2-3.4)	*n*=55	0.849	
ALT(U/L)	17(14-21)	16(13-19.7)	*n*=112	0.193	
GGT(U/L))	13(11-15)	12(11-14)	*n*=109	**0.035**	0.348

Data are median (interquartile range). SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol. MetS, Metabolic syndrome.HbA1C, glycosylated haemoglobin. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein. ALT, Alanine aminotransferase. GGT, gamma-glutamyl transferase.

Table 3. Differences between included children with and without follow-up from the Spanish Cohort

	Included without follow-up (n=553)	**Included with follow-up (n=173)**	**P value**	**P value for difference- Adjustment for age***
Age (years)	8.51(7.0-10.2)	6.5(6.0-8.0)	**<0.001**	
Sex % female/male	45.5/51.9	52/48	0.211	
Cardio metabolic risk variables				
Waist (cm)	67(55-78)	54.5(50.7-61.0)	**<0.001**	**<0.001**
SBP (mmHg)	108(100-115)	103(95-108)	**<0.001**	**<0.001**
DBP (mmHg)	61(56-66)	56(52-62)	**<0.001**	**<0.001**
Glucose (mg/dL)	87(83-92)	85(82-89)	**0.001**	0.2631
TG (mg/dL)	55(43-77)	51(42.5-62.5)	**0.003**	0.302
HDL-C (mg/dL)	54(46-63.2)	56.5(48-65)	0.074	
Insulin (ulU/ml)	5.05(2.35-9.16)	2.0(0.50-4.14)	**<0.001**	**<0.001**
HOMA-IR	1.08(0.50-2.04)	0.39(0.10-0.88)	**<0.001**	**<0.001**
HbA_{1c}(%)	5.3(5.2-5.5)	5.3(5.1-5.5)	0.160	
Iron markers				
Ferritin (µg/L)	39(27-54)	30.6(21.1-42.6)	**<0.001**	**0.005**
Transferrin (mg/dL)	276(253-300)	264(246-289)	**0.001**	**0.004**
Covariates				
Height (cm)				
BMI Z score	0.86(-0.49 to 1.93)	-0.16(-0.65 to 0.59)	**<0.001**	**<0.001**
CRP (mg/L)	87(83-92)	85(82-89)	**0.003**	0.756
ALT(U/L)	17(14-21)	17(14-20)	0.053	0.277
GGT(U/L)	13(11-16)	12(11-14)	**0.002**	0.4537

Data are median (interquartile range). SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol. MetS, Metabolic syndrome.HbA1C, glycosylated haemoglobin. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein. ALT, Alanine aminotransferase. GGT, gamma-glutamyl transferase.

Table 4. Relationship between age and sex-specific standardized values (or Z scores) of iron markers and MetS-related markers and insulin resistance at baseline (cross-sectional study) and their change (Δ) per year of follow-up (prospective study). [Spanish Cohort]

	Ferritin SD units at baseline (µg/L)								
	Model 1		Model 2			Model 1		Model 2	
	βeta (95% CI)	*P*	βeta (95% CI)	*P*		βeta (95% CI)	*P*	βeta (95% CI)	*P*
Glucose (mg/dL) SD	**-0.07 (-0.14 to 0.004)**	**0.038**	**-0.09 (-0.16 to -0.01)**	**0.017**	Δ / year	-0.02 (-0.06 to 0.009)	0.137	-0.02 (-0.06 to 0.01)	0.193
DBP(mmHg) SD*	0.003 (0.001 to 0.02)	0.186	-0.003 (-0.009 to 0.01)	0.163	Δ / year	0.0008 (-0.005 to 0.007)	0.801	0.0008 (-0.006 to 0.007)	0.822
SBP (mmHg) SD*	**0.01 (0.005 to 0.01)**	**<0.001**	0.003 (0.02 to 0.04)	0.263	Δ / year	**0.008 (0.001 to 0.01)**	**0.019**	0.007 (-0.0005 to 0.01)	0.070
log-TG (mg/dL) SD	**0.09 (0.01 to 0.16)**	**0.015**	0.03 (-0.03 to 0.10)	0.340	Δ / year	-0.03 (-0.07 to 0.0004)	0.052	-0.03 (-0.07 to 0.006)	0.097
HDL-C (mg/dL) SD	**-0.09 (-0.16 to -0.02)**	**0.009**	-0.03 (-0.10 to -0.03)	0.290	Δ / year	0.03 (-0.0008 to 0.06)	0.056	0.02 (-0.005 to 0.06)	0.104
log-Waist (cm) SD	**0.17 (0.10 to 0.24)**	**<0.001**	**0.06 (0.02 to 0.09)**	**0.002**	Δ / year	**-0.03 (-0.06 to -0.002)**	**0.036**	-0.02 (-0.05 to 0.004)	0.094
log-HOMA-IR SD	**0.08 (0.009 to 0.15)**	**0.028**	0.006 (-0.05 to 0.07)	0.837	Δ / year	-0.002 (-0.05 to 0.05)	0.942	0.02 (-0.03 to 0.08)	0.382
HbA_{1c}(%)SD	**-0.11 (-0.19 to -0.04)**	**0.002**	**-0.14 (-0.22 to -0.07)**	**<0.001**	Δ / year	-0.01 (-0.06 to 0.02)	0.447	-0.01 (-0.05 to 0.03)	0.589
	Transferrin SD units at baseline (mg/dL)								
Glucose (mg/dL) SD	0.01 (-0.06 to 0.08)	0.767	-0.001 (-0.07 to 0.07)	0.960	Δ / year	0.007 (-0.03 to 0.04)	0.693	0.019 (-0.02 to 0.06)	0.366
DBP(mmHg) SD*	0.003 (-0.001 to 0.08)	0.191	-0.003 (-0.008 to 0.001)	0.193	Δ / year	0.009 (-0.05 to 0.007)	0.774	0.0009 (-0.006 to 0.008)	0.798
SBP (mmHg) SD*	**0.01 (0.005 to 0.01)**	**<0.001**	0.003 (0.002 to 0.008)	0.250	Δ / year	**0.008 (0.01 to 0.01)**	**0.019**	0.007 (-0.0005 to 0.01)	0.071
log-TG (mg/dL)	**0.08**	**0.019**	0.03	0.314	Δ / year	-0.002	0.914	-0.01	0.479

SD	**(0.01 to 0.15)**		(-0.03 to 0.10)			(-0.04 to 0.03)		(-0.05 to 0.02)	
HDL-C (mg/dL) SD	0.04 (-0.02 to 0.11)	0.184	**0.10 (0.03 to 0.16)**	**0.004**	Δ / year	**-0.05 (-0.08 to -0.019)**	**0.002**	**-0.04 (-0.08 to -0.01)**	**0.006**
log-Waist (cm) SD	**0.11 (0.04 to 0.18)**	**0.002**	0.005 (-0.03 to 0.04)	0.773	Δ / year	0.03 (-0.0001 to 0.06)	0.051	0.02 (-0.003 to 0.06)	0.083
log-HOMA-IR SD	**0.17 (0.10 to 0.24)**	**<0.001**	**0.11 (0.04 to 0.17)**	**<0.001**	Δ / year	0.04 (-0.006 to 0.10)	0.083	0.02 (-0.02 to 0.08)	0.314
HbA_{1c}(%)SD	**0.11 (0.04 to 0.18)**	**0.002**	**0.09 (0.02 to 0.17)**	**0.008**	Δ / year	0.009 (-0.03 to 0.05)	0.705	0.008 (-0.04 to 0.05)	0.742

Model 1: adjusted for age and sex. Model 2: adjusted for model 1 plus CRP, ALT and GGT levels and BMI Z score. *Model 1 included also height. For the prospective associations, the above models also included the respective Z score of the outcome at baseline as covariate. Significant associations are shown in bold. Skewed variables were log- transformed [ferritin, CRP, ALT, GGT, WC(baseline),triglycerides and HOMA-IR], except CRP levels which were inverse log-transformed. SD, standard deviation units. SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol.HbA_{1C}, glycosylated haemoglobin. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein. ALT, Alanine aminotransferase. GGT, gamma-glutamyl transferase.

Table 5. Relationship between age and sex-specific standardized values (or Z scores) of iron markers and MetS-related markers and insulin resistance at baseline (cross-sectional study) and their change (Δ) per year of follow-up (prospective study) by sex. [Spanish Cohort] (3 pages table)

	Ferritin SD units at baseline (μg/L)								
	Model 1		Model 2			Model 1		Model 2	
Boys	βeta (95% CI)	*P*	βeta (95% CI)	*P*		βeta (95% CI)	*P*	βeta (95% CI)	*P*
Glucose (mg/dL) SD	-0.07 (-0.17 to 0.02)	0.136	**-0.10 (-0.20 to -0.004)**	**0.041**	Δ / year	-0.01 (-0.07 to 0.04)	0.590	-0.01 (-0.07 to 0.04)	0.548
DBP(mmHg) SD*	0.005 (-0.09 to 0.10)	0.920	-0.03 (-0.13 to 0.06)	0.498	Δ / year	0.001 (-0.007 to 0.01)	0.800	-0.00006 (-0.09 to 0.009)	0.989
SBP (mmHg) SD*	0.07 (-0.02 to 0.17)	0.133	0.04 (-0.05 to 0.14)	0.401	Δ / year	**0.01 (0.0005 to 0.01)**	**0.039**	0.007 (-0.002 to 0.01)	0.153
log-TG (mg/dL) SD	0.04 (-0.05 to 0.14)	0.376	-0.001 (-0.09 to 0.09)	0.983	Δ / year	-0.01 (-0.07 to 0.04)	0.661	-0.008 (-0.07 to 0.05)	0.791
HDL-C (mg/dL) SD	-0.03 (-0.13 to 0.06)	0.483	0.01 (-0.08 to 0.10)	0.806	Δ / year	**0.07 (0.02 to 0.11)**	**0.003**	**0.07 (0.02 to 0.11)**	**0.003**
log-Waist (cm) SD	**0.15 (005 to 0.25)**	**0.003**	**0.06 (0.05 to 0.11)**	**0.030**	Δ / year	**-0.04 (-0.09 to -0.001)**	**0.043**	-0.04 (-0.08 to 0.002)	0.061
log-HOMA-IR SD	**0.11 (0.01 to 0.21)**	**0.023**	0.04 (-0.04 to 0.13)	0.290	Δ / year	-0.03 (-0.11 to 0.03)	0.319	-0.008 (-0.08 to 0.07)	0.844
HbA_{1c}(%)SD	**-0.12 (-0.22 to -0.02)**	**0.013**	**-0.16 (-0.26to -0.06)**	**0.002**	Δ / year	0.003 (-0.06 to 0.07)	0.930	0.007 (-0.06 to 0.07)	0.836
Girls									
Glucose (mg/dL) SD	-0.07 (-0.18 to 0.02)	0.150	-0.07 (-0.18 to 0.03)	0.162	Δ / year	-0.03 (-0.09 to 0.01)	0.126	-0.02 (-0.07 to 0.03)	0.398
DBP(mmHg) SD*	**0.13 (0.02 to 0.23)**	**0.013**	0.08 (-0.01 to 0.19)	0.109	Δ / year	0.0005 (-0.009 to 0.01)	0.906	0.0008 (-0.009 to 0.01)	0.873
SBP (mmHg) SD*	0.07 (-0.02 to 0.18)	0.140	0.03 (-0.07 to 0.14)	0.503	Δ / year	0.006 (-0.004 to 0.01)	0.247	0.004 (-0.007 to 0.01)	0.489
log-TG (mg/dL) SD	**0.13 (0.03 to .0.24)**	**0.010**	0.07 (-0.02 to 0.17)	0.145	Δ / year	**-0.05 (-0.11 to -0.006)**	**0.027**	**-0.06 (-0.11 to -0.006)**	**0.028**

HDL-C (mg/dL) SD	**-0.16 (-0.26 to -0.05)**	**0.002**	-0.09 (-0.19 to 0.08)	0.072	Δ / year	-0.001 (-0.04 to 0.04)	0.937	-0.006 (-0.05 to 0.04)	0.781
log-Waist (cm) SD	**0.20 (0.09 to 0.30)**	**<0.001**	**0.05 (0.006 to 0.10)**	**0.027**	Δ / year	-0.01 (-0.06 to 0.02)	0.345	-0.01 (-0.05 to 0.02)	0.448
log-HOMA-IR SD	0.04 (-0.06 to 0.14)	0.418	-0.04 (-0.13 to -0.05)	0.372	Δ / year	0.04 (-0.04 to 0.12)	0.345	0.05 (-0.02 to 0.14)	0.180
HbA_{1c}(%)SD	**-0.10 (-0.21 to -0.0007)**	**0.048**	**-0.12 (-0.23 to 0.01)**	**0.021**	Δ / year	-0.03 (-0.09 to 0.02)	0.274	-0.03 (-0.09 to 0.02)	0.257
	Transferrin SD units at baseline (mg/dL)								
	Model 1		Model 2			Model 1		Model 2	
Boys	βeta (95% CI)	*P*	βeta (95% CI)	*P*		βeta (95% CI)	*P*	βeta (95% CI)	*P*
Glucose (mg/dL) SD	0.01 (-0.08 to 0.12)	0.704	-0.004 (-0.10 to 0.09)	0.932	Δ / year	0.04 (-0.01 to 0.09)	0.147	0.05 (-0.006 to 0.11)	0.081
DBP(mmHg) SD*	**0.13 (0.03 to 0.23)**	**0.007**	0.07 (-0.02 to 0.17)	0.146	Δ / year	0.0009 (-0.008 to 0.009)	0.840	0.0001 (-0.009 to 0.010)	0.977
SBP (mmHg) SD*	**0.11 (0.01 to 0.21)**	**0.022**	0.05 (-0.04to 0.15)	0.316	Δ / year	**0.01 (0.0005 to 0.01)**	**0.039**	0.007 (-0.002 to 0.01)	0.141
log-TG (mg/dL) SD	**0.13 (0.03 to 0.23)**	**0.010**	0.06 (-0.03 to 0.15)	0.221	Δ / year	0.0006 (-0.06 to 0.06)	0.982	-0.008 (-0.07 to 0.05)	0.785
HDL-C (mg/dL) SD	0.03 (-0.06 to 0.13)	0.492	**0.11 (0.01 to 0.20)**	**0.020**	Δ / year	-0.02 (-0.07 to 0.02)	0.284	-0.02 (-0.07 to 0.02)	0.314
log-Waist (cm) SD	**0.17 (0.07 to 0.27)**	**0.001**	0.03 (-0.02 to 0.08)	0.278	Δ / year	0.02 (-0.02 to 0.07)	0.365	0.01 (-0.02 to 0.06)	0.443
log-HOMA-IR SD	**0.16 (0.06 to 0.26)**	**0.001**	0.07 (-0.01 to 0.16)	0.088	Δ / year	0.03 (-0.03 to 0.10)	0.358	0.006 (-0.07 to 0.08)	0.863
HbA_{1c}(%)SD	**0.11 (0.01 to 0.21)**	**0.028**	0.08 (-0.01 to 0.18)	0.101	Δ / year	0.02 (-0.03 to 0.09)	0.401	0.03 (-0.03 to 0.10)	0.350
Girls									

Glucose (mg/dL) SD	0.001 (-0.10 to 0.10)	0.974	-0.006 (-0.11 to 0.10)	0.901	Δ / year	-0.03 (-0008 to 0.02)	0.279	-0.02 (-0.08 to 0.04)	0.506
DBP(mmHg) SD*	**0.17 (0.07 to 0.28)**	**0.001**	**0.16 (0.06 to 0.27)**	**0.002**	Δ / year	0.007 (-0.009 to 0.01)	0.880	0.0009 (-0.009 to 0.01)	0.866
SBP (mmHg) SD*	0.06 (-0.03 to 0.16)	0.224	0.05 (-0.04 to 0.16)	0.276	Δ / year	**0.006 (-0.004 to 0.01)**	**0.229**	0.004 (-0.007 to 0.01)	0.460
log-TG (mg/dL) SD	0.03 (-0.06 to 0.14)	0.480	0.01 (-0.08 to 0.10)	0.835	Δ / year	-0.009 (-0.06 to 0.05)	0.761	-0.03 (-0.09 to 0.03)	0.325
HDL-C (mg/dL) SD	0.06 (-0.04 to 0.16)	0.231	0.08 (-0.01 to 0.18)	0.085	Δ / year	**-0.08 (-0.12 to -0.03)**	**0.001**	**-0.07 (-0.12 to -0.02)**	**0.003**
log-Waist (cm) SD	0.05 (-0.05 to 0.15)	0.325	-0.01 (-0.06 to 0.03)	0.571	Δ / year	0.04 (-0.003 to 0.08)	0.069	0.03 (-0.007 to 0.08)	0.096
log-HOMA-IR SD	**0.17 (0.07 to 0.28)**	**0.001**	**0.14 (0.05 to 0.23)**	**0.002**	Δ / year	0.07 (-0.01 to 0.16)	0.107	0.05 (-0.03 to 0.14)	0.232
HbA_{1c}(%)SD	**0.11 (0.01 to 0.22)**	**0.028**	**0.11 (0.008 to 0.21)**	**0.035**	Δ / year	-0.01 (-0.07 to 0.05)	0.761	-0.01 (-0.09 to 0.05)	0.634

Model 1: adjusted for age. Model 2: adjusted for model 1 plus CRP, ALT and GGT levels and BMI Z score. *Model 1 included also height. For the prospective associations, the above models also included the respective Z score of the outcome at baseline as covariate. Significant associations are shown in bold. Skewed variables were log- transformed [ferritin, CRP, ALT, GGT, WC(baseline),triglycerides and HOMA-IR], except CRP levels which were inverse log-transformed. SD, standard deviation units. SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol.HbA_{1C}, glycosylated haemoglobin. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein. ALT, Alanine aminotransferase. GGT, gamma-glutamyl transferase.

Table 6. Differences between included and excluded children from the Chilean Cohort at the different points of follow-up

At 16-17 years	**Included (n=567) Valid sample with no missing values at 16-17 years**	**Excluded (n=*1223*)**	***n* for variables in excluded**	***P value***
Assigned to iron supplementation at infancy n(%)			*n=1223*	
High iron	269(47.4)	449(36.7)		**<0.001**
Low iron	14(2.5)	391(32)		
No iron	237(41.8)	297(24.3)		
No randomised	47(8.3)	86(7.0)		
Sex % female/male	47.6/52.4	46.2/53.8	*n=1223*	0.575
Ferritin (µg/L)	26.3(15.7-39)	24.8(15.3-35.1)	*n=352*	0.198
Haemoglobin (g/dL)	14.7(13.6-15.9)	14.5(13.4-15.5)	*n=353*	**<0.001**
CRP (mg/L)	0.42(0.13-1.29)	0.44(0.14-1.48)	*n=110*	0.830
BMI Z score	0.60(-0.10 to 1.26)	0.53(-0.29 to 1.24)	*n=112*	0.398
Tanner stage			*n=473*	
2	2(0.4)	2(0.4)		0.452
3	65(11.5)	40(8.5)		
4	313(55.2)	267(56.4)		
5	187(33)	164(34.7)		
Waist (cm)	78.7(73.2-87.6)	79.3(74.1-88)	*n=112*	0.465
SBP (mmHg)	110(104-120)	110(103-119)	*n=112*	0.471
DBP (mmHg)	70(65-74)	69(62-72.7)	*n=112*	0.564
Glucose (mg/dL)	88.1(82.6-94.2)	90.4(84.1-95.6)	*n=111*	0.284
TG (mg/dL)	74.2(57-101.1)	72.8(58.5-104.2)	*n=111*	0.819
HDL-C (mg/dL)	38.9(32.2-46.3)	40.2(32.8-47.5)	*n=111*	0.296
Insulin (ulU/ml)	6.83(4.64-10)	5.98(4.51-8.84)	*n=110*	0.138
HOMA-IR	1.45(0.98-2.17)	1.35(0.97-1.90)	*n=110*	0.187
At 5 years	**Included (n=565) Valid sample with no missing values at 5 and 16-17 years**	**Excluded (*n=1225*)**		
Assigned to iron supplementation at infancy n(%)			*n=1225*	
High iron	269(47.6)	449(36.7)		**<0.001**
Low iron	13(2.3)	392(32)		
No iron	236(41.8)	298(24.3)		
No randomised	47(8.3)	86(7.0)		
Sex % female/male	47.4/52.6	46.3/53.7	*n=1225*	0.651
Ferritin (µg/L)	21.6(14.6-30.4)	20.8(13.2-28.8)	*n=333*	0.161
Haemoglobin (g/dL)	12.9(12.5-13.5)	129(124-13.5)	*n=335*	0.808
BMI Z score	0.84(0.19 to 1.56)	0.75(0.08 to 1.49)	*n=323*	0.282

At 10 years	**Included (n=381) Valid sample with no missing values at 10 and 16-17 years**	**Excluded (n=*1409*)**		
Assigned to iron supplementation at infancy n(%)			*n=1409*	
High iron	206(54.1)	512(36.3)		**<0.001**
Low iron	13(3.4)	392(27.8)		
No iron	128(33.6)	406(28.8)		
No randomised	34(8.9)	99(7.0)		
Sex % female/male	48/52	53.7/46.3	*n=1409*	0.542
Ferritin (µg/L)	26.5(20.4-35.7)	27.4(19.5-37.4)	*n=507*	0.602
Haemoglobin (g/dL)	13.7(13.1-14.1)	136(13.1-14.1)	*n=689*	0.574
BMI Z score	0.90(0.13 to 1.50)	0.71(-0.02 to 1.38)	*n=745*	**0.043**
Tanner stage			*n=677*	
1	212(55.6)	369(54.5)		0.448
2	137(36)	257(38)		
3	31(8.1)	44(6.5)		
4	1(0.3)	3(0.4)		
SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein.				

Table 7. Alternative adjusted beta coefficients for variation in MetS Z score by combinations of high (H) and low/moderate (L/M) ferritin across intervals of the follow-up

	Girls		Boys	
	Beta (95% CI)	P value	Beta (95% CI)	P value
5-10 years[¥]				
L/M-L/M	0(Reference)		0(Reference)	
L/M-H	0.12(-0.08 to 0.34)	0.242	0.07(-0.13 to 0.29)	0.464
H-L/M	-0.06(-0.29 to 0.15)	0.546	0.04(-0.17 to 0.26)	0.681
H-H	-0.03(-0.23 to 0.15)	0.694	**0.28(0.06 to 0.49)**	**0.010**
10-16 years[¶]				
L/M-L/M	0(Reference)		0(Reference)	
L/M-H	0.09(-0.09 to 0.29)	0.312	0.02(-0.14 to 0.19)	0.801
H-L/M	0.04(-0.13 to 0.23)	0.629	0.01(-0.15 to 0.18)	0.843
H-H	-0.02(-0.21 to 0.16)	0.774	**0.32(0.14 to 0.50)**	**<0.001**
5-16 years[Ψ]				
L/M-L/M	0(Reference)		0(Reference)	

L/M-H	0.17(-0.002 to 0.36)	0.054	0.08(-0.09 to 0.26)	0.367
H-L/M	-0.01(-0.19 to 0.16)	0.857	-0.01(-0.20 to 0.16)	0.854
H-H	-0.16(-0.35 to 0.02)	0.089	**0.24(0.05 to 0.43)**	**0.011**

[¥] Beta are adjusted for BMI Z score and haemoglobin at 5 years, and the change in BMI Z score and haemoglobin during the interval. [¶] Beta are adjusted for BMI Z score, haemoglobin, and tanner stage at 10 years, and the change in BMI Z score, haemoglobin, and tanner stage during the interval. [Ψ] Beta are adjusted for BMI Z score, haemoglobin at 5 years, tanner stage at 16 years and the change in BMI and haemoglobin during the interval.

Table 8. Beta coefficients for MetS Z score by patterns of ferritin concentration combinations [high (H) and low/moderate (L/M)] across the three stages of follow-up

	Girls				Boys			
5-10-16-years	Unadjusted	P value	Adjusted*	P value	Unadjusted*	P value	Adjusted	P value
L/M-L/M-L/M (girls n =72; boys n=75)	0 (Reference)		0 (Reference)		0 (Reference)		0 (Reference)	
L/M-L/M-H (girls n =23; boys n=25)	0.22(-0.02 to 0.47)	0.082	0.14(-0.06 to 0.35)	0.171	-0.005(-0.27 to 0.26)	0.969	-0.024(-0.22 to 0.17)	0.790
L/M-H-L/M (girls n =18; boys n=24)	-0.01(-0.28 to 0.26)	0.932	0.01(-0.22 to 0.25)	0.902	0.006(-0.26 to 0.28)	0.964	-0.01(-0.22 to 0.19)	0.883
L/M-H-H (girls n =8; boys n=8)	0.48(0.09 to 0.86)	0.015	0.28(-0.04 to 0.62)	0.087	**0.62(0.18 to 1.05)**	**0.006**	0.29(-0.02 to 0.62)	0.073
H-L/M-L/M (girls n =19; boys n=22)	0.05(-0.21 to 0.32)	0.698	-0.07(-0.30 to 0.14)	0.489	-0.001(-0.28 to 0.28)	0.989	-0.08(-0.30 to 0.12)	0.424
H-L/M-H (girls n =6; boys n=10)	-0.22(-0.67 to 0.21)	0.308	-0.20(-0.57 to 0.16)	0.282	0.35(-0.03 to 0.75)	0.077	0.09(-0.20 to 0.39)	0.512
H-H-L/M (girls n =14; boys n=11)	0.13(-0.17 to 0.43)	0.308	0.04(-0.21 to 0.29)	0.743	0.36(-0.01 to 0.74)	0.059	0.06(-0.21 to 0.35)	0.648
H-H-H (girls n =21; boys n=23)	-0.07(-0.32 to 0.18)	0.588	-0.15(-0.36 to 0.06)	0.166	**0.45(0.17 to 0.73)**	**0.002**	**0.31(0.11 to 0.52)**	**0.003**
*Adjusted for BMI Z score at 10 and 16 years, tanner stage at 10 and 16 years , and haemoglobin at 10 and 16 years								

Table 9. Sex-stratified relationships of ferritin level Z scores at the three points of follow-up with Z scores of MetS-related markers and insulin resistance (as Z score) at 16-17 years by sex. [Chilean Cohort]

	Girls				Boys			
	Unadjusted		Adjusted		Unadjusted		Adjusted	
	Beta(95% CI)	P value	Beta(95% CI)	P value	Beta(95% CI)	P value	Beta(95% CI)	P value
At 5 yearsψ								
Z SBP (mmHg)*	-0.01(-0.13 to 0.11)	0.852	-0.01(-0.12 to 0.10)	0.854	0.03(-0.07 to 0.14)	0.538	0.007(-0.09 to 0.11)	0.890
Z DBP (mmHg)*	0.006(-0.11 to 0.13)	0.920	0.01(-0.11 to 0.13)	0.851	0.01(-0.09 to 0.12)	0.831	-0.009(-0.11 to 0.10)	0.864
Z Glucose (mg/dL)	-0.03(-0.16 to 0.087)	0.544	-0.04(0.16 to 0.08)	0.503	0.07(-0.03 to 0.18)	0.163	0.07(-0.03 t0 0.19)	0.162
Z TG (mg/dL)	-0.09(-0.22 to 0.02)	0.131	-0.10(-0.22 to 0.02)	0.113	0.08(-0.02 to 0.19)	0.124	0.05(-0.04 to 0.16)	0.285
Z HDL-C (mg/dL)	-0.08(-0.20 to 0.04)	0.192	0.08(-0.04 to 0.20)	0.205	**-0.24(-0.35 to 0.13)**	**<0.001**	**-0.22(-0.33 to 0.11)**	**<0.001**
Z Waist (cm)	0.03(-0.09 to 0.16)	0.586	0.02(-0.07 to 0.12)	0.593	**0.12(0.01 to 0.23)**	**0.022**	0.07(-0.01 to 0.16)	0.103
Z HOMA-IR	0.03(-0.09 to 0.15)	0.601	0.03(-0.09 to 0.15)	0.617	**0.12(0.01 to 0.23)**	**0.030**	0.08(-0.01 to 0.19)	0.099
At 10 years¶								
Z SBP (mmHg)*	-0.01(-0.15 to 0.12)	0.844	-0.03(-0.16 to 0.09)	0.598	0.05(-0.09 to 0.20)	0.463	0.01(-0.11 to 0.15)	0.794
Z DBP (mmHg)*	-0.08(-0.23 to 0.05)	0.245	-0.08(-0.22 to 0.05)	0.211	-0.03(-0.17 to 0.10)	0.643	-0.04(-0.18 to 0.09)	0.548
Z Glucose (mg/dL)	-0.01(-0.16 to 0.13)	0.846	-0.01(-0.17 to 0.13)	0.807	0.12(-0.02 to 0.27)	0.109	*0.13(-0.01 to 0.28)*	*0.077*
Z TG (mg/dL)	0.04(-0.09 to 0.19)	0.537	0.03(-0.11 to 0.17)	0.674	0.11(-0.01 to 0.25)	0.090	0.08(-0.04 to 0.21)	0.214
Z HDL-C (mg/dL)	-0.02(-0.15 to 0.11)	0.732	-0.006(-0.14 to 0.13)	0.924	**-0.16(-0.30 to 0.02)**	**0.021**	*-0.13(-0.27 to 0.006)*	*0.062*
Z Waist (cm)	0.08(-0.06 to 0.22)	0.282	0.04(-0.06 to 0.15)	0.432	0.08(-0.06 to 0.22)	0.259	-0.01(-0.11 to 0.08)	0.785
Z HOMA-IR	**0.17(0.02 to 0.31)**	**0.025**	**0.14(0.002 to 0.29)**	**0.046**	0.12(-0.02 to 0.26)	0.094	0.85(-0.05 to 0.22)	0.229
At 16-17 years¥								
Z SBP (mmHg)*	0.009(-0.10 to 0.12)	0.868	-0.05(-0.16 to 0.05)	0.316	0.13(-0.01 to 0.27)	0.070	0.03(-0.10 to 0.17)	0.643
Z DBP (mmHg)*	-0.0004(-0.11 to 0.11)	0.994	-0.06(-0.17 to 0.05)	0.314	0.10(-0.03 to 0.35)	0.154	0.05(-0.09 to 0.20)	0.474

Z Glucose (mg/dL)	0.09(-0.02 to 0.20)	0.128	0.08(-0.04 to 0.20)	0.205	**0.15(0.009 to 0.29)**	**0.037**	**0.19(0.045 to 0.34)**	**0.010**
Z TG (mg/dL)	0.07(-0.04 to 0.19)	0.199	0.02(-0.93 to 0.15)	0.640	**0.27(0.12 to 0.41)**	**<0.001**	**0.16(0.03 to 0.30)**	**0.015**
Z HDL-C (mg/dL)	0.09(-0.01 to 0.21)	0.102	*0.11(-0.003 to 0.24)*	*0.058*	0.03(-0.11 to 0.17)	0.658	0.12(-0.01 to 0.26)	0.078
Z Waist (cm)	**0.12(0.008 to 0.24)**	**0.035**	0.04(-0.02 to 0.11)	0.199	**0.23(0.09 to 0.37)**	**<0.001**	0.01(-0.05 to 0.08)	0.596
Z HOMA-IR	**0.18(-0.06 to 0.29)**	**0.002**	**0.14(0.02 to 0.25)**	**0.014**	**0.25(0.11 to 0.40)**	**<0.001**	**0.18(0.05 to 0.31)**	**0.004**

ψ Estimates were adjusted for BMI Z score and haemoglobin level. ¶ Estimates were adjusted for BMI Z score, haemoglobin level and tanner stage. ¥ Estimates were adjusted for BMI Z score, CRP level, haemoglobin level and tanner stage. *Adjustment model also included height. Significant associations are shown in bold. SBP, systolic blood pressure. DBP, diastolic blood pressure. TG, triglycerides. HDL-C, HDL cholesterol. HOMA-IR, homeostatic model assessment insulin resistance. BMI, body mass index. CRP, C reactive protein.

Table 10. Differences between included and excluded subjects of the VIKING

	Included (*n*=2047)	Excluded (*n*=89)	*n* for variables in excluded	P value
Age (years)	51(38.9-63.2)	51.2(41-55.9)	*n*=87	0.696
Pre-MW/ Post-MW/ Men %	28.8/30.5/40.7	36.2/28.8/34	*n*=47	
BMI (kg/m^2)	26.7(24.1-29.9)	27.0(23.7-31.4)	*n*=81	0.457
Ferritin(µg/L)	56(29-97)	47(23-75)	*n*=57	**0.043**
Fibrinogen (g/L)	3.35(2.91-3.89)	3.24(2.82-4.16)	*n*=57	0.683
GGT U/L	16(12-24)	14(11-19)	*n*=55	**0.041**
ALT U/L	22(17-29)	21(17-27)	*n*=55	0.268
AST U/L	22(19-26)	22(19-26)	*n*=55	0.387
HDL-C (mmol/L)	1.46(1.22-1.75)	1.61(1.30-1.79)	*n*=57	**0.026**
SBP (mmHg)	127(116-142)	127(117-143)	*n*=81	0.898
DBP (mmHg)	75(69-82)	77(69-84)	*n*=81	0.227
WC (cm)	91.2(81.5-100.8)	91.5(77.9-104.1)	*n*=81	0.843
Glucose (mmol/L)	4.8(4.5-5.1)	4.7(4.4-5.0)	*n*=57	0.200
TG (mmol/L)	0.90(0.60-1.30)	0.80(0.50-1.00)	*n*=57	0.067
Insulin mU/mL	37.1(25.8-54)	35.4(22.9-48)	*n*=57	0.264
HOMA-IR	7.83(5.37-11.82)	7.20(4.80-10.24)	*n*=57	0.199
HbA1C (%)	5.30(5.10-5.50)	5.40(5.20-5.50)	*n*=57	0.136
Smoking n(%)			*n*=83	
Never smoker	1133(55.3)	44(53)		0.678
ex-regular or ex-occasional smoker	747(36.5)	30(36.1)		
Current smoker	167(8.2)	9(10.8)		
Alcohol consumption n(%)			*n*=78	
Non-drinker or ex-drinker	84(4.1)	6(7.7)		0.107
trivial drinker	152(7.4)	9(11.5)		
drinker	1811(88.5)	63(80.8)		
Cardiovascular disease n(%)	64(3.1)	5(5.6)	*n*=89	0.157
Diabetes n(%)	46(2.2)	2(2.2)	*n*=87	0.975

Data are median (interquartile range) or n(%). Comparison between groups by Mann-Whitney U and χ^2 test. Pre-MW, premenopausal women. Post-MW, postmenopausal women. BMI, body mass indexTG, triglycerides. SBP, systolic blood pressure. DBP, diastolic blood pressure. WC, waist circumference. HOMA-IR, homeostatic model assessment insulin resistance. Trivial drinker (special occasions or 1 to 3 days per month). Drinker (1 or more days per week) ; SheS 95-98: 1) non-drinker or ex-drinker, 2) trivial drinker < 1 rating unit per week and 3) drinker ≥ 1 rating unit per week.

Table 11. Differences between included and excluded subjects of the SHeS 1995 and 1998 (Cross-sectional analysis Chapter 3)

	Included (*n*=8653)	Excluded (*n*=8619)	*n* for variables in excluded	P value
Age (years)	42(32-54)	40(28-56)	*n*=8619	**<0.001**
Pre-MW/ Post-MW/ Men %	33.6/20.1/46.3	33/22.6/44.4	*n*=8110	**<0.001**
BMI (kg/m^2)	25.6(23.1-28.7)	25.5(22.4-29.1)	*n*=7052	**0.001**
Ferritin(µg/L)	60(32-106)	59(32-29.1)	*n*=3347	0.779
Fibrinogen (g/L)	3.0(2.5-3.6)	3.1(2.6-3.8)	*n*=2614	**<0.001**
GGT U/L	20(15-32)	21(15-34)	*n*=3524	**0.005**
HDL-C (mmol/L)	1.4 (1.2-1.7)	1.4(1.1-1.6)	*n*=3277	**<0.001**
SBP (mmHg)	127(117-139)	127(116-140)	*n*=3925	0.328
DBP (mmHg)	71(64-79)	70(62-78)	*n*=3925	**<0.001**
WC (cm)	85(76-94.3)	85.3(75.3-95.6)	*n*=5327	0.148
Smoking n(%)			*n*=8287	
Never smoker	3349(38.7)	3013(36.4)		**<0.001**
ex-regular or ex-occasional smoker	2376(27.5)	1939(23.4)		
Current smoker	2928(33.8)	3335(40.2)		
Alcohol consumption n(%)			*n*=8215	
Non-drinker or ex-drinker	687(7.9)	958(11.1)		**<0.001**
trivial drinker	1001(11.6)	9(13.8)		
drinker	6965(80.5)	63(74.6)		
Cardiovascular disease n(%)	206(2.4)	434(5.0)	*n*=8319	**<0.001**
Diabetes n(%)	153(1.8)	241(2.8)	*n*=8318	**<0.001**

Data are median (interquartile range) or n(%). Comparison between groups by Mann-Whitney U and χ^2 test. Pre-MW, premenopausal women. Post-MW, postmenopausal women. BMI, body mass index. TG, triglycerides. SBP, systolic blood pressure. DBP, diastolic blood pressure. WC, waist circumference. HOMA-IR, homeostatic model assessment insulin resistance. Trivial drinker < 1 rating unit per week. Drinker ≥ 1 rating unit per week.

Table 12. Unadjusted odds ratios (95% CI) for cardiometabolic risk markers by sex- and menopausal-specific quartiles (Q) of serum ferritin in the study cohorts

	VIKING (2013-2015)					**Scottish Health Surveys (1995 & 1998)**				
	Q1	**Q2**	**Q3**	**Q4**	**P for trend**	**Q1**	**Q2**	**Q3**	**Q4**	**P for trend**
Premenopausal women	≤ 15 µg/L	16-25 µg/L	26-41 µg/L	> 41 µg/L		2-18 µg/L	19-30 µg/L	31-47 µg/L	48-950 µg/L	
Low HDL-C	1.0 (Ref)	0.95 (0.54-1.65)	0.84 (0.48-1.47)	**1.91 (1.14-3.19)**	**0.020**	1.0 (Ref)	0.86 (0.64-1.17)	1.00 (0.75-1.33)	1.19 (0.90-1.58)	0.140
High SBP and/or high DBP	1.0 (Ref)	0.69 (0.37-1.30)	0.86 (0.47-1.57)	1.45 (0.82-2.55)	0.132	1.0 (Ref)	0.56 (0.42-0.77)	0.65 (0.48-0.86)	0.90 (0.68-1.18)	0.597
High WC	1.0 (Ref)	0.70 (0.44-1.12)	0.80 (0.50-1.28)	1.46 (0.90-2.36)	0.108	1.0 (Ref)	0.86 (0.67-1.10)	0.96 (0.76-1.23)	1.22 (0.96-1.55)	0.066
High fasting glucose	1.0 (Ref)	4.05 (0.44-36.7)	2.0 (0.17-22.2)	5.17 (0.59-44.8)	0.196	1.0 (Ref)				
High TG	1.0 (Ref)	1.52 (0.52-4.38)	1.16 (0.38-3.55)	1.54 (0.53-4.45)	0.554	1.0 (Ref)				
Postmenopausal women	≤ 34 µg/L	35-54 µg/L	55-84 µg/L	> 84 µg/L		3- 35 µg/L	36-59 µg/L	60-92 µg/L	93-1000 µg/L	
Low HDL-C	1.0 (Ref)	0.47 (0.25-0.89)	0.77 (0.44-1.34)	1.07 (0.63-1.81)	0.495	1.0 (Ref)	0.92 (0.63-1.33)	1.30 (0.91-1.86)	**1.68 (1.19-2.37)**	**0.001**
High SBP and/or high DBP	1.0 (Ref)	0.59 (0.37-0.94)	1.22 (0.76-1.96)	1.22 (0.76-1.95)	0.083	1.0 (Ref)	0.93 (0.68-1.26)	0.81 (0.60-1.09)	**1.46 (1.07-1.98)**	0.055
High WC	1.0 (Ref)	1.14 (0.70-1.85)	2.32 (1.36-3.94)	**3.19 (1.81-5.62)**	**<0.001**	1.0 (Ref)	1.25 (0.92-1.69)	1.56 (1.15-2.12)	**2.24 (1.64-3.06)**	**<0.001**
High fasting glucose	1.0 (Ref)	1.05 (0.42-2.61)	0.91 (0.36-2.30)	1.66 (0.73-3.79)	0.254	1.0 (Ref)				

High TG	1.0 (Ref)	0.50 (0.20-1.20)	1.39 (0.69-2.77)	1.82 (0.94-3.54)	**0.012**	1.0 (Ref)				
Men	≤ 58 µg/L	59-89 µg/L	90-138 µg/L	>138 µg/L		3- 61 µg/L	62-96 µg/L	97-152 µg/L	153-2251 µg/L	
Low HDL-C	1.0 (Ref)	1.31 (0.65-2.65)	1.37 (0.68-2.77)	**2.21 (1.15-4.24)**	**0.015**	1.0 (Ref)	0.94 (0.71-1.26)	1.04 (0.78-1.39)	**1.61 (1.22-2.11)**	**0.001**
High SBP and/or high DBP	1.0 (Ref)	0.99 (0.67-1.47)	0.89 (0.60-1.33)	1.43 (0.95-2.15)	0.137	1.0 (Ref)	1.00 (0.82-1.22)	1.20 (0.98-1.47)	**1.79 (1.46-2.20)**	**<0.001**
High WC	1.0 (Ref)	1.10 (0.74-1.62)	1.37 (0.92-2.04)	**2.62 (1.71-4.01)**	**<0.001**	1.0 (Ref)	1.35 (1.09-1.67)	1.60 (1.30-1.97)	**2.98 (2.42-3.67)**	**<0.001**
High fasting glucose	1.0 (Ref)	1.02 (0.52-2.00)	0.76 (0.37-1.58)	**2.39 (1.32-4.34)**	**0.004**	1.0 (Ref)				
High TG	1.0 (Ref)	0.78 (0.44-1.37)	1.29 (0.76-2.18)	**1.79 (1.08-2.96)**	**0.005**	1.0 (Ref)				

Table 13. Adjusted* odds ratios (95% CI) for MetS components by sex- and menopausal-specific quartiles (Q) of serum ferritin in the study cohorts

		Viking Health study –Shetland (2013-2015)				Scottish Health Surveys (1995 & 1998)			
	Q1	**Q2**	**Q3**	**Q4**	**P for trend**	**Q2**	**Q3**	**Q4**	**P for trend**
Premenopausal women		16-25 µg/L	26-41 µg/L	> 41-67.1 µg/L		19-30 µg/L	31-47 µg/L	48-950 µg/L	
Low HDL-C	1.0 (Ref)	0.88 (0.49-1.58)	0.70 (0.38-1.28)	1.35 (0.77-2.35)	0.377	0.82 (0.59-1.13)	0.92 (0.67-1.24)	1.02 (0.74-1.39)	0.735
High SBP and/or high DBP	1.0 (Ref)	0.74 (0.37-1.48)	0.96 (0.48-1.89)	1.11 (0.58-2.10)	0.590	0.64 (0.46-0.88)	0.70 (0.51-0.94)	0.83 (0.61-1.12)	0.275
High WC	1.0 (Ref)	0.52 (0.25-1.10)	0.64 (0.30-1.36)	0.50 (0.23-1.10)	0.156	1.01 (0.69-1.46)	1.01 (0.70-1.46)	1.05 (0.71-1.53)	0.804
High fasting glucose	1.0 (Ref)	3.57 (0.35-35.7)	1.52 (0.12-18.6)	2.59 (0.27-24.8)	0.654				
High TG	1.0 (Ref)	1.51 (0.48-4.71)	0.94 (0.27-3.24)	0.85 (0.26-2.77)	0.580				
Postmenopausal women		35-54 µg/L	55-82 µg/L	> 84-450 µg/L		36-59 µg/L	60-92 µg/L	93-1000 µg/L	
Low HDL-C	1.0 (Ref)	0.52 (0.27-1.00)	0.59 (0.33-1.08)	0.99 (0.56-1.75)	0.994	0.93 (0.63-1.37)	1.33 (0.91-1.94)	**1.64 (1.11-2.43)**	**0.004**
High SBP and/or high DBP	1.0 (Ref)	0.54 (0.31-0.96)	0.76 (0.42-1.36)	0.57 (0.32-1.03)	0.164	0.83 (0.59-1.17)	0.59 (0.42-0.84)	0.89 (0.63-1.28)	0.226
High WC	1.0 (Ref)	2.66 (1.19-5.92)	2.25 (0.95-5.33)	**4.10 (1.65-10.2)**	**0.005**	1.53 (0.96-2.43)	1.58 (0.98-2.54)	1.56 (0.95-2.56)	0.081
High fasting glucose	1.0 (Ref)	1.27 (0.49-3.25)	0.69 (0.26-1.81)	1.44 (0.61-3.43)	0.623				
High TG	1.0 (Ref)	0.57 (0.23-1.44)	1.04 (0.50-2.19)	1.60 (0.79-3.24)	0.094				
Men		35-55 µg/L	90-138 µg/L	>138 -772 µg/L		62-96 µg/L	97-152 µg/L	153-2251	

								µg/L	
Low HDL-C	1.0 (Ref)	1.43 (0.70-2.95)	1.35 (0.65-2.78)	1.81 (0.91-3.61)	0.114	0.88 (0.66-1.19)	0.88 (0.65-1.19)	1.20 (0.88-1.63)	0.255
High SBP and/or high DBP	1.0 (Ref)	1.08 (0.65-1.79)	0.67 (0.40-1.12)	0.86 (0.51-1.45)	0.266	0.87 (0.71-1.08)	0.96 (0.77-1.20)	1.09 (0.85-1.39)	0.380
High WC	1.0 (Ref)	1.07 (0.57-2.00)	0.83 (0.44-1.58)	1.39 (0.70-2.77)	0.555	1.14 (0.84-1.54)	1.13 (0.84-1.54)	**1.42 (1.06-1.90)**	**0.025**
High fasting glucose	1.0 (Ref)	1.17 (0.56-2.44)	0.71 (0.32-1.56)	1.91 (0.98-3.74)	0.089				
High TG	1.0 (Ref)	0.83 (0.46-1.51)	1.17 (0.67-2.03)	1.27 (0.74-2.18)	0.222				
* In both studies adjustment for age, glutamyl-transferase levels, fibrinogen levels, smoking, alcohol consumption, and BMI. In the VIKING the adjustment also included alanine-aminotransferase levels, aspartate-aminotransferase levels, Ref, reference. Significant associations are shown in bold P<0.05). Q, quartile.									

Table 14. Unadjusted and adjusted odds ratios (95% CI) for MetS components by SD units of serum ferritin (log-transformed) in the study cohorts

	VIKING (2013-2015)				Scottish Health Surveys (1995 & 1998)			
	Unadjusted		Adjusted*		Unadjusted		Adjusted*	
	Ferritin Z score	P value	Ferritin Z score	P value	Ferritin Z score	P value	Ferritin Z score	P value
Premenopausal women								
Low HDL-C	**1.23 (1.05-1.49)**	**0.034**	1.05 (0.86-1.30)	0.586	1.07 (0.96-1.19)	0.187	1.02 (0.90-1.16)	0.689
High SBP and/or high DBP	1.16 (0.94-1.44)	0.157	1.00 (0.79-1.27)	0.958	0.96 (0.86-1.07)	0.475	0.90 (0.81-1.00)	0.069
High WC	**1.19 (1.01-1.41)**	**0.034**	0.81 (0.62-1.07)	0.155	**1.10 (1.00-1.20)**	0.029	1.03 (0.90-1.18)	0.593
High fasting glucose	**1.97 (104-3.74)**	**0.036**	1.38 (0.73-2.68)	0.312				
High TG	1.25 (0.86-1.82)	0.157	1.00 (0.67-1.48)	0.998				
Postmenopausal women								
Low HDL-C	1.07 (0.87-1.31)	0.503	1.00 (0.80-1.25)	0.943	**1.23 (1.08-1.41)**	**0.002**	**1.20 (1.03-1.40)**	**0.013**
High SBP and/or high DBP	1.11 (0.94-1.30)	0.208	0.81 (0.65-1.01)	0.062	**1.16 (1.05-1.29)**	**0.004**	0.99 (0.88-1.12)	0.968
High WC	**1.56 (1.29-1.88)**	**<0.001**	**1.53 (1.11-2.12)**	**0.009**	**1.38 (1.23-1.54)**	**<0.001**	**1.22 (1.02-1.46)**	**0.023**
High fasting glucose	**1.39 (1.008-1.93)**	**0.044**	1.28 (0.91-1.79)	0.142				
High TG	**1.33 (1.02-1.73)**	**0.030**	1.21 (0.92-1.60)	0.168				
Men								
Low HDL-C	**1.32 (1.04-1.66)**	**0.020**	1.20 (0.95-1.52)	0.118	**1.19 (1.06-1.32)**	**0.001**	1.05 (0.93-1.18)	0.380
High SBP and/or high DBP	1.06 (0.95-1.22)	0.379	0.90 (0.74-1.09)	0.291	**1.24 (1.15-1.33)**	**<0.001**	1.02 (0.93-1.12)	0.587
High WC	**1.34 (1.15-1.55)**	**<0.001**	1.09 (085-1.40)	0.481	**1.54(1.42-1.67)**	**<0.001**	**1.16 (1.04-1.29)**	**0.005**
High fasting glucose	**1.27 (1.01-1.59)**	**0.038**	1.13 (0.90-1.42)	0.275				
High TG	**1.24 (1.03-1.50)**	**0.021**	1.08 (0.89-1.31)	0.413				

* In both studies adjustment for age, glutamyl-transferase levels, fibrinogen levels, smoking, alcohol consumption, and BMI. In the VIKING the adjustment also included alanine-aminotransferase levels, aspartate-aminotransferase levels, Ref, reference. Significant associations are shown in bold ($p<0.05$)

Table 15. Beta coefficients (95% CI) for values of glycosylated haemoglobin (HbA1C) by ferritin levels (log-transformed) in subjects from VIKING

	log-HbA1C		log-HOMA-IR	
	Unadjusted	Adjusted*	Unadjusted	Adjusted*
Premenopausal women	**-0.02(-0.03 to -0.007)**	**-0.03 (-0.04 to -0.01)**	0.09 (-0.03 to 0.15)	-0.01 (-0.06 to 0.03)
Postmenopausal women	-0.01 (-0.02 to 0.003)	**-0.03 (-0.04 to- 0.01)**	0.10 (0.03 to 0.16)	0.003 (-0.05 to 0.05)
Men	**-0.03 (0.04 to -0.01)**	**-0.03 (-0.05 to -0.02)**	**0.09 (0.03 to 0.16)**	-0.02 (-0.08 to 0.02)
All**	-0.005(-0.02 to -0.01)	**-0.03 (-0.04 to -0.02)**	**0.12(0.09 to 0.15)**	-0.01 (-0.04 to 0.01)

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes), and BMI ** Additionally adjusted for sex/menopausal status (premenopausal women/ postmenopausal women/men). Multivariate analyses were performed on transformed values of skewed variables: logarithm of sTfR , ferritin , HOMA-IR values, body mass index and fibrinogen values; square of age and square root of glycosylated haemoglobin. Significant associations are show in bold (P<0.05).

Table 16. Information about exclusion criteria for factors of possible influence in exposure (iron markers) and the outcome (metabolic syndrome)

	Exposure					Outcome	
Study	**Pregnancy/ nursing**	**Inflammatory processes**	**Hepatic disorders**	**Anaemia/iron deficiency**	**Other Iron disorders**	**Type 2 diabetes**	**Cardiovascular disease**
Jehn et al., 2004	Yes	Yes	Yes	Yes	Yes (high levels of multiple iron parameters)	No	No
Vari et al.,2007 (Mets in baseline as exlclusion factor no mentioned)	No	Yes (high CRP)	No	Yes , very low ferritin	Yes high ferritin (>400 and 300)	No	No
Zelber-Sagi et al., 2007	Yes	No	Yes (NAFLD)	No	No	No	No
Shi et al., 2008	Yes	No	No	Not apply (anaemia was an exposure variable)	No	No	No
Sun et al., 2008	No	Yes	No	No	Yes(ferritin>500 μg/L)	No	No
Cho et al. ,2011	No	No	No(but transaminases level was a covariate)	No	No	No	No
Kim et al. , 2011	No	Yes	Yes	Yes	No	No	Yes
Lee et al.,2001	Yes	No	Yes	Yes(by recent	Yes(ferritin>500 μg/L)	No	No

	Exposure					Outcome	
Study	**Pregnancy/ nursing**	**Inflammatory processes**	**Hepatic disorders**	**Anaemia/iron deficiency**	**Other Iron disorders**	**Type 2 diabetes**	**Cardiovascular disease**
				treatment)			
Ryoo et al. ,2011 (Malignancy excluded)	Not apply (only men)	No(but an inflammatory marker was covariate)	Yes(hep B)	No	Yes(ferr>800)	Yes	Yes(Hypertension excluded by treatment)
Park et al., 2012 (MetS, Malignancy excluded and lipid lowering drugs)	Not apply (only men)	No(but an inflammatory marker was covariate)	Yes(hep B and C, and high ALT)	No	Yes(ferritin>800 µg/L and transfusions history)	No(but diabetes status was covariate)	Yes
Yoon et al. , 2012 (MetS Malignancy excluded)	No	No (but an inflammatory marker was covariate)	No (but transaminases level was a covariate)	No	No	Yes (on the base of anti-diabetic drugs as exclusion criterion at baseline)	Yes (on the basis of anti-hypertension, and lowering-lipids drugs s as exclusion criteria at baseline)
Chang et al.,2013	No	No (but an inflammatory marker was covariate)	No(but transferases level was a covariate)	No(but it was covariate)	Yes(ferritin >500 µg/L)	No	No
Li et al. , 2013	No	No(but an inflammatory marker was covariate)	No	No	No	No	No

	Exposure					Outcome	
Study	**Pregnancy/ nursing**	**Inflammatory processes**	**Hepatic disorders**	**Anaemia/iron deficiency**	**Other Iron disorders**	**Type 2 diabetes**	**Cardiovascular disease**
Kilani et al., 2014	No	No(but an inflammatory marker was covariate)	No(but transferases level was a covariate)	No	Yes (TSAT >50%)	No	No
Ledesma et al., 2015	No apply (only men)	Yes (high CRP)	No	No	Yes (very high iron status)	No	No
Seo et al.2015	No apply (only postmenopausal women)	No	No	No	Yes(transfusions history)	No	Yes (cardiovascular disease)
Tang et al., 2015	No apply (only men)	No	Yes (liver disease)	No	Yes(high ferritin)	No	No
Kilani et al., 2015	No	No(but an inflammatory marker was covariate)	No(but transferases level was a covariate)	No	Yes (TSAT >50%)	No	No
Hamalainen et al.,2012	No	No(but an inflammatory marker was covariate)	No	No	No	No	No
Martinelli et al.,2012	No	No(but an inflammatory marker was covariate)	No	No	Yes (c282Y HFE mutation n=7)	No	No
Hamalainen et al.,2014	No	No(but an inflammatory marker was covariate)	No	No	No	No	No
Iwanaga et	No	No	No	No	No	Yes	Yes

	Exposure					Outcome	
Study	**Pregnancy/ nursing**	**Inflammatory processes**	**Hepatic disorders**	**Anaemia/iron deficiency**	**Other Iron disorders**	**Type 2 diabetes**	**Cardiovascular disease**
al. 2014							
VIKING	No	Yes (but as a sensitivity analysis)	Yes (but as a sensitivity analysis)	Yes (but as a sensitivity analysis)	Yes (High ferritin – but as a sensitivity analysis)	No (but diabetes status was an extra-adjustment)	No (but cardiovascular disease status was an extra-adjustment)
SHeS 95-98	Yes	Yes (but as a sensitivity analysis)	Yes (but as a sensitivity analysis)	Yes (but as a sensitivity analysis)	Yes (High ferritin – but as a sensitivity analysis)	No (but diabetes status was an extra-adjustment)	No (but cardiovascular disease status was an extra-adjustment)
TSAT, transferrin saturation. CRP, C reactive protein. NAFLD, Non-alcoholoic fatty liver disease.							

Table 17. Tertiles and quartiles of reported values for average ferritin levels and cut-offs for high ferritin levels (cross-sectional and prospective studies)

Study	year	Ferritin levels ug/L (mean or median)
tertile 1		
VHS-Shetland study(Pre-MW)	2016	25
Yoon et al.(Pre-MW)	2012	26.7
Cho et al.(Pre-MW)	2011	30.2
VHS-Shetland study(Post-MW)	2016	55
Seo et al. (Post-MW)	2015	63.1
Cho et al. (Post-MW)	2011	64.7
Shi et al. (W)	2008	46.7
VHS-Shetland study(M)	2016	90
Shi et al. (M)	2008	110.8
Park et al. (M)	2012	112.5
Li et al. (M)	2013	121.9
Zelber-Sagi et al	2007	73.4
tertile 2		
Lee et al. (Pre-MW)	2011	33.9
Jehn et al. (Pre-MW)	2004	37
Yoon et al.(Post-MW)	2012	67.1
Lee et al. (Post-MW)	2011	70.5
Vari et al. (Post-MW)	2007	91.7
Li et al. (W)	2013	51
Lee et al. (M)	2011	122
Yoon et al.(M)	2012	128.9
Jehn et al. (M)	2004	146
Kim et al. (M)	2011	156
Sun et al.	2008	133.8
tertile 3		
Kilani et al.(Pre-MW)	2015	54
Vari et al.(Pre-MW)	2007	56.4
Jehn et al. (Post-MW)	2004	96
Kilani et al. (Post-MW)	2015	113
Kim et al. (W)	2011	59
Ledesma et al.(M)	2015	174
Vari et al. (M)	2007	178
Kilani et al. (M)	2015	208
Tang et al. (M)	2015	301.1
Chang et al.	2013	173

Study	year	Ferritin levels ug/L (mean or median)
quartile 1		
VHS-Shetland study(Pre-MW)	2016	25
Yoon et al.(Pre-MW)	2012	26.7
VHS-Shetland study(Post-MW)	2016	55
Seo et al. (Post-MW)	2015	63.1
VHS-Shetland study(M)	2016	90
Shi et al. (M)	2008	110.8
Park et al. (M)	2012	112.5
quartile 2		
Cho et al.(Pre-MW)	2011	30.2
Lee et al. (Pre-MW)	2011	33.9
Cho et al. (Post-MW)	2011	64.7
Yoon et al.(Post-MW)	2012	67.1
Shi et al. (W)	2008	46.7
Li et al. (M)	2013	121.9
Lee et al. (M)	2011	122
Yoon et al.(M)	2012	128.9
Zelber-Sagi et al	2007	73.4
quartile 3		
Jehn et al. (Pre-MW)	2004	37
Kilani et al.(Pre-MW)	2015	54
Lee et al. (Post-MW)	2011	70.5
Vari et al. (Post-MW)	2007	91.7
Li et al. (W)	2013	51
Jehn et al. (M)	2004	146
Kim et al. (M)	2011	156
Ledesma et al.(M)	2015	174
Sun et al.	2008	133.8
quartile 4		
Vari et al.(Pre-MW)	2007	56.4
Jehn et al. (Post-MW)	2004	96
Kilani et al. (Post-MW)	2015	113
Kim et al. (W)	2011	59
Vari et al. (M)	2007	178
Kilani et al. (M)	2015	208
Tang et al. (M)	2015	301.1
Chang et al.	2013	173

Study	year	Cut-off point (ug/L) reported for high ferritin levels
tertile 1		
Yoon et al.(Pre-MW)	2012	36.3
VHS-Shetland study(Pre-MW)	2016	41
Cho et al. (Post-MW)	2011	51.58
VHS-Shetland study(Post-MW)	2016	82
Yoon et al.(Post-MW)	2012	83.1
Kim et al. (W)	2011	86
VHS-Shetland study(M)	2016	138
Lee et al. (M)	2011	150
Park et al. (M)	2012	155.8
Yoon et al.(M)	2012	164.3
Zelber-Sagi et al	2007	100
tertile 2		
Lee et al. (Pre-MW)	2011	46
Cho et al.(Pre-MW)	2011	51.58
Lee et al. (Post-MW)	2011	85
Seo et al. (Post-MW)	2015	110.8
Li et al. (W)	2013	93.2
Shi et al. (M)	2008	175.6
Kim et al. (M)	2011	209
Li et al. (M)	2013	213
Jehn et al. (M)	2004	231
Chang et al.	2013	176.4
tertile 3		
Jehn et al. (Pre-MW)	2004	60
Kilani et al.(Pre-MW)	2015	90
Jehn et al. (Post-MW)	2004	168
Kilani et al. (Post-MW)	2015	171
Shi et al. (W)	2008	95.6
Ledesma et al.(M)	2015	261
Kilani et al. (M)	2015	326
Tang et al. (M)	2015	426.6
Sun et al.	2008	188.1

Study	year	Cut-off point (ug/L) reported for high ferritin levels
quartile 1		
Yoon et al.(Pre-MW)	2012	36.3
VHS-Shetland study(Pre-MW)	2016	41
Cho et al. (Post-MW)	2011	51.58
VHS-Shetland study(Post-MW)	2016	82
VHS-Shetland study(M)	2016	138
Lee et al. (M)	2011	150
Park et al. (M)	2012	155.8
quartile 2		
Lee et al. (Pre-MW)	2011	46
Yoon et al.(Post-MW)	2012	83.1
Lee et al. (Post-MW)	2011	85
Kim et al. (W)	2011	86
Yoon et al.(M)	2012	164.3
Shi et al. (M)	2008	175.6
Kim et al. (M)	2011	209
Zelber-Sagi et al	2007	100
quartile 3		
Cho et al.(Pre-MW)	2011	51.58
Jehn et al. (Pre-MW)	2004	60
Seo et al. (Post-MW)	2015	110.8
Jehn et al. (Post-MW)	2004	168
Li et al. (W)	2013	93.2
Li et al. (M)	2013	213
Jehn et al. (M)	2004	231
Ledesma et al.(M)	2015	261
Chang et al.	2013	176.4
quartile 4		
Kilani et al.(Pre-MW)	2015	90
Kilani et al. (Post-MW)	2015	171
Shi et al. (W)	2008	95.6
Kilani et al. (M)	2015	326
Tang et al. (M)	2015	426.6
Sun et al.	2008	188.1

Pre-MW, premenopausal women. Post-MW, Postmenopausal women. M, men. Cut-off values defining the tertiles for average ferritin levels (percentiles 33.3th and 66.6th) were for premenopausal women group 31.4 and 48.3 µg/L; for post-menopausal women 66.3 and 93.1 µg/L; for studies with category of women 48.1 and 56.3 µg/L; for men 122 and 174 µg/L; and for studies describing levels in both sexes 93.5 and 159.9 µg/L.

Cut-off values defining the quartiles for average ferritin levels (percentiles 25th, 50th, and 75th) were for premenopausal women group 29.3, 35.4, and 54.6 µg/L; for post-menopausal women 64.7, 70.5, and 96 µg/L; for men 121.9, 146, and 178 µg/L; for studies with category of women 46.7 (25th percentile) and 51 µg/L (50 th percentile), 75th percentile was missing (few valuess); and for studies describing levels in both sexes 73.4(25th percentile) and 133.8 µg/L(50 th percentile), 75th percentile was missing (few values)

Cut-off values for the tertiles of cut-offs defining high ferritin (percentiles 33.3th and 66.6th) were: for premenopausal women group 46 and 60 µg/L; for post-menopausal women 83.7 and 148 µg/L; for studies with category of women 88.4 and 94.8 µg/L; for men 171.8 and 241 µg/L; and for studies describing levels in both sexes 125.4 and 184.4 µg/L.

Cut-off values for the tertiles of cut-offs defining high ferritin (percentiles 25th, 50th, and 75th) were: for premenopausal women group 41.1, 51.5, and 75 µg/L; for post-menopausal women 75.2, 97.9, and 168.7 µg/L; for men 162.1, 211 and 277.2 µg/L; for studies with category of women 86(25th percentile) and 93.2 µg/L (50th percentile), 75th percentile was missing (few values); and for studies describing levels in both sexes 100(25th percentile) and 176.4 µg/L(50th percentile), 75th percentile was missing (few valuess) .

Table 18. Tertiles and quartiles of reported values for average ferritin levels and cut-offs for high ferritin levels (cross-sectional studies only)

Study	year	Ferritin levels ug/L (mean or median)
tertile 1		
VHS-Shetland study(Pre-MW)	2016	25
SHS 1995-1998 study(Pre-MW)	2016	30
Cho et al.(Pre-MW)	2011	30.2
VHS-Shetland study(Post-MW)	2016	55
SHS 1995-1998 study(Post-MW)	2016	56
Seo et al. (Post-MW)	2015	63.1
Cho et al. (Post-MW)	2011	64.7
Shi et al. (W)	2008	46.7
VHS-Shetland study(M)	2016	90
SHS 1995-1998 study(M)	2016	92
Shi et al. (M)	2008	110.8
Li et al. (M)	2013	121.9
Lee et al. (M)	2011	122
Zelber-Sagi et al	2007	73.4
tertile 2		
Lee et al. (Pre-MW)	2011	33.9
Jehn et al. (Pre-MW)	2004	37
Lee et al. (Post-MW)	2011	70.5
Vari et al. (Post-MW)	2007	91.7
Li et al. (W)	2013	51
Jehn et al. (M)	2004	146
Ryoo et al. (M)	2011	152.6
Kim et al. (M)	2011	156
Ledesma et al.(M)	2015	174
Sun et al.	2008	133.8
tertile 3		
Kilani et al. (Pre-MW)	2014	54
Vari et al. (Pre-MW)	2007	56.4
Jehn et al. (Post-MW)	2004	96
Kilani et al. (Post-MW)	2014	113
Kim et al. (W)	2011	59
Vari et al. (M)	2007	178
Kilani et al. (M)	2014	208
Tang et al.(M)	2015	316.8
Chang et al.	2013	173

Study	year	Ferritin levels ug/L (mean or median)
quartile 1		
VHS-Shetland study(Pre-MW)	2016	25
SHS 1995-1998 study(Pre-MW)	2016	30
Cho et al.(Pre-MW)	2011	30.2
VHS-Shetland study(Post-MW)	2016	55
SHS 1995-1998 study(Post-MW)	2016	56
Seo et al. (Post-MW)	2015	63.1
VHS-Shetland study(M)	2016	90
SHS 1995-1998 study(M)	2016	92
Shi et al. (M)	2008	110.8
Li et al. (M)	2013	121.9
quartile 2		
Lee et al. (Pre-MW)	2011	33.9
Cho et al. (Post-MW)	2011	64.7
Lee et al. (Post-MW)	2011	70.5
Shi et al. (W)	2008	46.7
Lee et al. (M)	2011	122
Jehn et al. (M)	2004	146
Ryoo et al. (M)	2011	152.6
Zelber-Sagi et al	2007	73.4
quartile 3		
Jehn et al. (Pre-MW)	2004	37
Kilani et al. (Pre-MW)	2014	54
Vari et al. (Post-MW)	2007	91.7
Jehn et al. (Post-MW)	2004	96
Li et al. (W)	2013	51
Kim et al. (M)	2011	156
Ledesma et al.(M)	2015	174
Vari et al. (M)	2007	178
Sun et al.	2008	133.8
quartile 4		
Vari et al. (Pre-MW)	2007	56.4
Kilani et al. (Post-MW)	2014	113
Kim et al. (W)	2011	59
Kilani et al. (M)	2014	208
Tang et al.(M)	2015	316.8
Chang et al.	2013	173

Study	year	Cut-off point (ug/L) reported for high ferritin levels
tertile 1		
VHS-Shetland study(Pre-MW)	2016	41
Lee et al. (Pre-MW)	2011	46
SHS 1995-1998 study(Pre-MW)	2016	47
Cho et al. (Post-MW)	2011	51.58
VHS-Shetland study(Post-MW)	2016	82
Kim et al. (W)	2011	86
VHS-Shetland study(M)	2016	138
SHS 1995-1998 study(M)	2016	149
Lee et al. (M)	2011	150
Shi et al. (M)	2008	175.6
Kim et al. (M)	2011	209
Zelber-Sagi et al	2007	100
tertile 2		
Cho et al.(Pre-MW)	2011	51.58
Jehn et al. (Pre-MW)	2004	60
Lee et al. (Post-MW)	2011	85
SHS 1995-1998 study(Post-MW)	2016	90
Seo et al. (Post-MW)	2015	110.8
Li et al. (W)	2013	93.2
Ryoo et al. (M)	2011	212.8
Li et al. (M)	2013	213
Jehn et al. (M)	2004	231
Chang et al.	2013	176.4
tertile 3		
Kilani et al. (Pre-MW)	2014	90
Jehn et al. (Post-MW)	2004	168
Kilani et al. (Post-MW)	2014	171
Shi et al. (W)	2008	95.6
Ledesma et al.(M)	2015	261
Kilani et al. (M)	2014	326
Tang et al.(M)	2015	459.9
Sun et al.	2008	188.1

Study	year	Cut-off point (ug/L) reported for high ferritin levels
quartile 1		
VHS-Shetland study(Pre-MW)	2016	41
Lee et al. (Pre-MW)	2011	46
SHS 1995-1998 study(Pre-MW)	2016	47
Cho et al. (Post-MW)	2011	51.58
VHS-Shetland study(Post-MW)	2016	82
VHS-Shetland study(M)	2016	138
SHS 1995-1998 study(M)	2016	149
Lee et al. (M)	2011	150
Shi et al. (M)	2008	175.6
quartile 2		
Cho et al.(Pre-MW)	2011	51.58
Lee et al. (Post-MW)	2011	85
SHS 1995-1998 study(Post-MW)	2016	90
Kim et al. (W)	2011	86
Kim et al. (M)	2011	209
Ryoo et al. (M)	2011	212.8
Zelber-Sagi et al	2007	100
quartile 3		
Jehn et al. (Pre-MW)	2004	60
Seo et al. (Post-MW)	2015	110.8
Jehn et al. (Post-MW)	2004	168
Li et al. (W)	2013	93.2
Li et al. (M)	2013	213
Jehn et al. (M)	2004	231
Ledesma et al.(M)	2015	261
Chang et al.	2013	176.4
quartile 4		
Kilani et al. (Pre-MW)	2014	90
Kilani et al. (Post-MW)	2014	171
Shi et al. (W)	2008	95.6
Kilani et al. (M)	2014	326
Tang et al.(M)	2015	459.9
Sun et al.	2008	188.1

Pre-MW, premenopausal women. Post-MW, Postmenopausal women. M, men. Cut-off values defining the tertiles for average ferritin levels (percentiles 33.3th and 66.6th) were: for premenopausal women group 33.9 and 54 µg/L; for post-menopausal women 66.6 and 94.5 µg/L; for studies with category of women 48.1 and 56.3 µg/L; for men 138 and 175 µg/L; and for studies describing levels in both sexes 93.5 and 159.9 µg/L.

Cut-off values defining the quartiles for average ferritin levels (percentiles 25th, 50th, and 75th) were for premenopausal women group 32, 37, and 55.2 µg/L; for post-menopausal women 66.3, 81.1, and 100.2 µg/L; for men 121.9, 154.3, and 185.5 µg/L; for studies with category of women 46.7 (25th percentile) and 51 µg/L(50th percentile), 75th percentile was missing (few valuess) : and for studies describing levels in both sexes 73.4(25th percentile) and 133.8 (75th percentile) µg/L, 75th percentile was missing (few values).

Cut-off values for the tertiles of cut-offs defining high ferritin (percentiles 33.3th and 66.6th) were: for premenopausal women group 49.7 and 70 µg/L; for post-menopausal women 85 and 168 µg/L; for studies with category of women 88.4 and 94.8 µg/L; for men 210.2 and 251 µg/L; and for studies describing levels in both sexes 125.4 and 184.4 µg/L.

Cut-off values for the quartiles of cut-offs defining high ferritin (percentiles 25th, 50th, and 75th) were: for premenopausal women group 47.3, 55.7, and 82.5 µg/L; for post-menopausal women 68.2, 110.8, and 169.5 µg/L; for men 192.3, 213 and 293.5 µg/L; for studies with category of women 86(25th percentile) and 93.2 µg/L(50th percentile), 75th percentile was missing (few values): and for studies describing levels in both sexes 100(25th percentile) and 176.4 µg/L(50th percentile), 75th percentile was missing (few values).

Table 19. Results of the risk of bias assessment in Case-control and cross-sectional studies

First author, year	Selection			Exposure		Outcome	Compara-bility	Total points
	1) Represen-tativeness	2) Selection of controls	3) Definition of controls	1) Ascertain-ment of exposure	2) Same method for cases and controls	1) Case definition	1) Confoun-ding	
Jehn et al., 2004	1	1	1	1	1	1	1	7
Vari et al., 2007	1	1	1	0	1	1	0	5
Zelber-Sagi et al., 2007	1	1	1	0	1	0	0	4
Shi et al., 2008	1	1	1	0	1	1	0	5
Sun et al., 2008	1	1	1	1	1	1	1	7
Cho et al. ,2011	1	1	1	0	1	0	0	4
Kim et al., 2011	0	1	1	0	1	0	0	3
Lee et al.,2001	1	1	1	0	1	1	0	5
Ryoo et al. ,2011	0	1	1	0	1	0	0	3
Chang et al.,2013	1	1	1	0	1	0	0	4
Li et al. , 2013	1	1	1	0	1	0	0	4
Kilani et al. 2014	1	1	1	0	1	0	0	4
Ledesma et al., 2015	0	1	1	0	1	1	0	4
Seo et al., 2015	0	1	1	1	1	0	0	4
Tang et al., 2015	1	1	1	0	1	0	0	4
Hamalainen et al., 2012	0	1	1	0	1	1	0	4

First author, year	Selection			Exposure		Outcome	Compara-bility	Total points
	1) Represen-tativeness	2) Selection of controls	3) Definition of controls	1) Ascertain-ment of exposure	2) Same method for cases and controls	1) Case definition	1) Confoun-ding	
Martinelli et al.,2012	1	1	1	0	1	0	0	4
Iwanaga et al. 2014	0	1	1	0	1	0	0	3
VHS-Shetland study,2016	1	1	1	1	1	0	1	6
SHS 1995-1998 study, 2016	1	1	1	1	1	1	1	7

Table 20. Results of the risk of bias assessment in prospective studies

Cohort studies and nested case-control studies	Selection		Exposure	Outcome			Compara-bility	Total points
	1) Represen-tativeness	2) Absence of outcome at baseline	1) Ascertain-ment of exposure	1) Outcome Assessment	2) Follow up length	3) Follow-up rate	1) Confoun-ding	
Vari et al., 2007	1	0	1	1	1	0	0	4
Yoon et al., 2012	1	1	0	0	1	0	0	3
Park et al., 2012	0	1	0	0	1	1	0	3
Tang et al., 2015	1	1	0	0	1	1	0	4
Kilani et al., 2015	1	1	1	0	1	1	1	6
Hamalainen et al.,2014	0	1	0	1	1	1	1	5

Table 21. Stratified odds ratio for association between ferritin metabolic syndrome in the cross-sectional studies (meta-analysis 2)

Subgroup	Number Of studies*	O.R (95% CI)	I^2	P Value (meta-regression)
Region				
Asia	16 (10)	1.86(1.55-2.22)	75.5	
Europe	11 (5)	1.28(0.96-1.71)	77.3	
America	3 (1)	2.22(1.60-3.09)	7.5	0.437
Adjusted for BMI				
No	**11 (7)**	**2.04(1.65-2.52)**	**79.2**	
Yes	**19 (9)**	**1.45(1.20-1.76)**	**71.1**	**0.030**
Adjusted for CRP				
No	19 (11)	1.77(1.62-1.92)	0.1	
Yes	11 (5)	1.58(1.07-2.33)	91.7	0.769
Adjusted for some Inflammatory Marker				
No	15 (9)	1.02(1.16-2.04)	0.0	
Yes	15 (7)	1.54(1.16-2.04)	89.0	0.475
Adjusted for some hepatic function marker				
No	**13 (7)**	**2.09(1.74-2.50)**	**73.3**	
Yes	**17 (9)**	**1.35(1.11-1.65)**	**67.8**	**0.003**
Ferritin assay				
RIA	*10(4)*	*2.08(1.60-2.71)*	*76.3*	
QLA	*5 (4)*	*1.72 (1.37-2.15)*	*56.2*	
TIA	*9 (5)*	*1.23(0.86-1.76)*	*82.8*	
Other	*6 (3)*	*1.64 (1.38-1.94)*	*0.0*	*0.065*
Sample size				
<1000	8 (4)	1.63(1.36-1.94)	79.2	
≥1000	7 (4)	1.66(1.37-2.01)	84.0	0.918

Subgroup	Number Of studies*	O.R (95% CI)	I^2	P Value (meta-regression)
Risk of bias				
score < median	15(10)	1.63(1.25-2.12)	88.4	
score ≥ median	15 (6)	1.69(1.49-1.92)	16.2	0.896
Mean/median ferritin levels (Sex/menopausal-specific tertiles)				
Tertile 1	11	1.64(1.17-2.30)	82.1	
Tertile 2	9	2.01(1.84-2.21)	81.6	
Tertile 3	10	1.33(0.95-1.86)	0.0	0.273
Mean/median ferritin levels (Sex/menopausal-specific quartiles)				
Quartile 1	7	1.68(0.99-2.86)	86.9	
Quartile 2	8	1.82(1.62-2.05)	0.0	
Quartile 3	9	1.94(1.68-2.25)	33.2	
Quartile 4	6	1.25(0.81-1.96)	86.1	0.269
Cut-off point reported for highest category of ferritin levels (Sex/menopausal-specific tertiles)				
Tertile 1	10	1.36(1.17-1.60)	0.0	
Tertile 2	9	2.22(1.76-2.80)	76.5	
Tertile 3	8	1.39(0.96-2.01)	86.2	0.868
Cut-off point reported for highest category of ferritin levels (Sex/menopausal-specific quartiles)				
Quartile 1	7	1.37(1.13-1.67)	0.0	
Quartile 2	6	1.65(1.35-2.01)	40.5	
Quartile 3	8	2.38(1.83-3.10)	74.0	
Quartile 4	6	1.22(0.77-1.94)	88.1	0.809
** First number is taking into account sex/menopausal status groups from each study, and second number (in parenthesis) means number of studies.*				

Table 22. Stratified odds ratio for association between ferritin and components of metabolic syndrome

	High fasting glucose			High waist circumference			High triglycerides			Low HDL-C			High blood pressure		
Subgroup	**O.R (95% CI)**	**I^2(%)**	**P Value ***	**O.R (95% CI)**	**I^2(%)**	**P Value ***	**O.R (95% CI)**	**I^2(%)**	**P Value ***	**O.R (95% CI)**	**I^2(%)**	**P Value ***	**O.R (95% CI)**	**I^2(%)**	**P Value ***
Region															
Asia	1.70 (1.42-2.03)	81.5		1.66 (1.30-2.11)	74.7		2.16 (1.62-2.89)	91.6		1.62 (1.34-1.94)	74.9		1.09 (0.93-1.26)	66.5	
Europe	1.40 (1.24-1.59)	0.0		1.48 (1.26-1.75)	47.5		1.67 (1.41-1.97)	19.4		1.36 (1.18-1.57)	22.4		1.07 (0.93-1.22)	43.0	
America	2.15 (1.11-4.15)	74.3	0.542	1.36 (0.64-2.88)	78.3	0.310	2.46 (1.63-3.71)	35.9	0.691	1.14 (0.87-1.50)	0.0	0.054	0.98 (0.65-1.48)	0.0	0.669
Adjusted for BMI															
No	1.77 (1.39-2.25)	83.2		1.72 (1.41-2.09)	73.3		2.05 (1.54-2.74)	90.8		1.61 (1.29-2.01)	79.3		**1.21 (1.11-1.32)**	**24.2**	
Yes	1.63 (1.37-1.94)	68.4	0.609	1.39 (1.14-1.69)	55.6	0.170	1.88 (1.50-2.35)	63.9	0.580	1.37 (1.22-1.53)	13.0	0.171	**0.95 (0.83-1.08)**	**34.2**	**0.011**
Adjusted for CRP															
No	1.53 (1.37-1.72)	74.7		1.50 (1.33-1.70)	33.8		**1.74 (1.52-2.00)**	**58.0**		1.40 (1.28-1.54)	20.6		1.05 (0.95-1.17)	51.3	
Yes	2.03 (1.47-2.81)	88.4	0.067	1.60 (1.04-2.48)	87.5	0.587	**2.97 (2.26-3.91)**	**64.5**	**0.002**	1.47 (1.04-2.10)	81.9	0.525	1.21 (1.01-1.44)	23.2	0.341
Adjusted for some Inflammatory Marker															
No	149 (1.29-1.71)	74.7		1.60 (1.44-1.77)	0.0		1.81 (1.52-2.17)	64.3		1.52 (1.37-1.70)	0.0		1.10 (0.95-1.28)	56.7	
Yes	1.95	80.8	0.065	1.46	81.2	0.710	2.13	89.4	0.238	1.37	70.4	0.347	1.05	41.1	0.543

	High fasting glucose			High waist circumference			High triglycerides			Low HDL-C			High blood pressure		
Subgroup	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***
	(1.54-2.48)			(1.10-1.94)			(1.51-3.00)			(1.14-1.64)			(0.93-1.19)		
Adjusted for some hepatic injury marker															
No	1.84 (1.44-2.35)	83.4		1.62 (1.29-2.03)	77.4		2.23 (1.71-2.92)	88.6		1.57 (1.29-1.90)	72.0		1.17 (1.09-1.26)	0.0	
Yes	1.54 (1.35-1.74)	44.5	0.234	1.45 (1.21-1.73)	48.5	0.454	1.68 (1.34-2.11)	64.5	0.099	1.36 (1.21-1.52)	11.1	0.191	0.96 (0.80-1.14)	62.9	0.094
Ferritin assay															
RIA	1.99 (1.49-2.66)	78.8		1.48 (1.09-2.03)	80.9		2.23 (1.58-3.15)	86.9		1.47 (1.18-1.83)	71.1		0.99 (0.78-1.24)	64.6	
QLA	1.55 (1.29-1.87)	76.8		1.73 (1.41-2.14)	0.0		2.15 (1.53-3.01)	86.9		1.55 (1.18-2.03)	69.4		1.16 (1.05-1.28)	0.0	
TIA	1.53 (1.23-1.91)	0.0		1.51 (0.78-2.94)	78.4		1.65 (1.15-2.37)	41.6		1.23 (0.98-1.53)	0.0		0.98 (0.64-1.49)	68.5	
Other	134 (1.15-1.56)	0.0	0.073	1.45 (1.28-1.64)	0.0	0.746	1.56 (1.30-1.88)	0.0	0.078	1.43 (1.18-1.73)	42.6	0.607	1.07 (0.95-1.21)	13.2	0.896
Sample size															
<1000	1.37 (1.18-1.58)	0.0		1.49 (1.17-1.90)	52.1		**1.51 (1.28-1.79)**	**0.0**		1.56 (1.29-1.87)	0.0		1.08 (0.91-1.28)	28.1	
≥1000	1.76 (1.49-2.07)	80.3	0.331	1.54 (1.29-1.84)	71.1	0.841	**2.21 (1.77-2.77)**	**87.4**	**0.037**	1.43 (1.25-1.63)	66.3	0.586	1.08 (0.96-1.20)	54.1	0.927
Risk of bias															
score < median	1.76 (1.44-2.16)	84.9		**1.86 (1.50-2.32)**	**72.5**		**2.37 (1.77-3.17)**	**91.4**		1.63 (1.28-2.08)	83.3		**1.23 (1.11-1.37)**	**36.6**	

	High fasting glucose			High waist circumference			High triglycerides			Low HDL-C			High blood pressure		
Subgroup	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***	**O.R (95% CI)**	***I²*(%)**	**P Value ***
score ≥ median	2.59 (0.27-24.80)	59.0	0.564	**1.37 (1.17-1.60)**	**45.9**	**0.039**	**1.67 (1.41-1.96)**	**35.6**	**0.034**	1.35 (1.22-1.50)	0.0	0.152	**0.97 (0.87-1.09)**	**27.8**	**0.010**
Mean/ median ferritin levels (Sex/meno pausal-specific tertiles)															
Tertile 1	2.00 (1.40-2.86)	58.5		1.52 (1.10-2.11)	79.0		1.60 (0.88-2.92)	90.5		1.44 (1.08-1.92)	77.4		1.00 (0.82-1.22)	61.4	
Tertile 2	1.59 (1.34-189)	63.5		1.45 (1.17-1.81)	59.6		1.94 (1.58-2.39)	72.7		1.35 (1.23-1.48)	0.0		1.03 (0.88-1.22)	57.2	
Tertile 3	1.69 (1.31-2.19)	85.0	0.575	1.58 (1.38-1.81)	0.0	0.959	2.20 (1.66-2.92)	72.7	0.341	1.63 (1.40-1.90)	0.0	0.606	1.20 (1.06-1.35)	0.0	0.255
Mean/ median ferritin levels (Sex/meno pausal-specific quartiles)															
Quartile 1	2.57 (2.04-3.23)	0.0		1.51 (1.01-2.26)	83.9		1.77 (0.80-3.94)	88.0		1.45 (1.04-2.03)	80.2		0.98 (0.78-1.24)	66.9	
Quartile 2	1.61 (1.40-1.85)	5.9		1.21 (0.94-1.55)	15.4		1.62 (1.37-1.92)	36.1		1.31 (1.17-1.47)	0.0		0.91 (0.70-1.18)	64.5	
Quartile 3	1.68	84.1		1.59	23.8		2.05	75.2		1.41	0.9		1.17	0.0	

	High fasting glucose			High waist circumference			High triglycerides			Low HDL-C			High blood pressure		
Subgroup	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***
	(1.27-2.21)			(1.38-1.83)			(1.56-2.70)			(1.24-1.60)			(1.04-1.31)		
Quartile 4	1.59 (1.25-2.02)	75.1	0.344	1.67 (1.40-1.99)	0.0	0.535	2.45 (2.06-2.90)	0.0	0.430	1.76 (1.46-2.13)	0.0	0.497	1.25 (1.07-1.46)	0.0	0.109
Cut-off point reported for highest category of ferritin levels (Sex/menopausal-specific tertiles)															
Tertile 1	1.40 (1.27-1.56)	0.0		1.33 (1.02-1.73)	51.6		1.44 (1.18-1.77)	0.0		1.24 (1.07-1.43)	0.0		0.88 (0.73-1.07)	34.5	
Tertile 2	2.06 (1.66-2.54)	68.7		1.61 (1.20-2.15)	82.0		2.27 (1.63-3.17)	92.0		1.55 (1.28-1.88)	75.4		1.11 (0.96-1.28)	56.3	
Tertile 3	1.84 (1.09-3.12)	88.8	0.247	1.83 (1.47-2.27)	0.0	0.280	2.34 (1.95-2.82)	0.0	**0.043**	1.37 (0.94-1.99)	54.2	0.397	1.28 (1.06-1.54)	0.0	**0.043**
Cut-off point reported for highest category of ferritin levels (Sex/menopausal-specific															

	High fasting glucose			High waist circumference			High triglycerides			Low HDL-C			High blood pressure		
Subgroup	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***	**O.R (95% CI)**	***I*²(%)**	**P Value ***
quartiles)															
Quartile 1	1.49 (1.21-1.84)	0.0		1.35 (0.95-1.93)	59.5		1.46 (1.18-1.81)	0.0		1.30 (1.11-1.53)	0.0		**0.88 (0.70-1.13)**	**42.2**	
Quartile 2	1.47 (1.31-1.65)	27.0		1.27 (1.00-1.63)	0.0		1.61 (1.45-1.79)	0.0		1.31 (1.10-1.56)	35.0		**0.92 (0.73-1.15)**	**66.2**	
Quartile 3	2.26 (1.75-2.92)	75.8		1.68 (1.28-2.19)	81.5		2.74 (2.20-3.40)	71.6		1.47 (1.15-1.88)	77.5		**1.28 (1.15-1.42)**	**2.0**	
Quartile 4	1.25 (0.94-1.67)	--	0.243	2.04 (1.22-3.41)	--	0.224	2.45 (1.73-3.46)	--	**0.004**	2.15 (1.27-3.63)	--	0.168	**1.11 (0.80-1.54)**	**--**	**0.028**
**P values are from meta-regression analysis.*															

Table 23. Differences between included and excluded subjects of the CROATIA-VIS STUDY

	Included (*n*=725)	Excluded (*n*=304)	*n* for variables in excluded	P value
Age (years)	57(46-69.5)	55(45-67)	*n*=193	0.275
Pre-MW/ Post-MW/ Men %	20.8/40/39.2	22/30.9/47	*n*=304	**0.018**
BMI (kg/m^2)	27.1(24.1-30)	26.7(24-30.2)	*n*=293	0.920
sTfR (mg/L)	3.1(2.6-3.8)	3.0(2.7-145.1)	*n*=49	0.881
Ferritin(µg/L)	76.6(36.7-140.2)	73(42.9-145.1)	*n*=102	0.571
Fibrinogen (g/L)	3.7(3.1-4.3)	3.5(2.9-4.2)	*n*=278	**0.024**
HDL-C (mmol/L)	1.14(1.0-1.23)	1.15(1.0-1.23)	*n*=283	0.642
SBP (mmHg)	135(120-152.2)	130(120-149)	*n*=293	0.063
DBP (mmHg)	80(72-87)	80(72-87)	*n*=293	0.752
WC (cm)	96.7(86.7-103.5)	95.6(87.2-104)	*n*=292	0.932
Glucose (mmol/L)	5.4(5.0-6.0)	5.3(4.8-6.1)	*n*=286	0.290
TG (mmol/L)	1.4(1.1-2.0)	1.4(1.2-2.0)	*n*=286	0.179
Insulin mU/mL	7.0(4.0-10)	6.0(4.0-10)	*n*=281	0.928
HOMA-IR	1.55(0.97-2.51)	1.52(0.92-2.47)	*n*=281	0.688
HbA1C (%)	5.3(5.0-5.6)	5.3(5.6)	*n*=279	0.242
Smoking n(%)			*n*=299	
Yes	196(27)	95(31.8)		0.187
No	315(43.4)	113(37.8)		
Ex-smoker	214(29.5)	91(30.4)		
Alcohol consumption Yes n(%)	308(42.5)	176(57.9)/113(37.2)	*n*=289	0.324
Cardiovascular disease n(%)	102(4.1)	41(13.5)	*n*=304	0.439
Diabetes n(%)	50(6.9)	17(5.6)	*n*=304	0.805

Data are median (interquartile range) or n(%). Comparison between groups by Mann-Whitney U and χ^2 test. Pre-MW, premenopausal women. Post-MW, postmenopausal women. BMI, body mass index. sTfR, soluble transferrin receptor. TG, triglycerides. SBP, systolic blood pressure. DBP, diastolic blood pressure. WC, waist circumference. HOMA-IR, homeostatic model assessment insulin resistance.

Table 24. Odds ratios(95% CI) for MetS components per sex/menopausal-specific SD units of the iron markers in the study subjects categorised by sex and menopausal status

	Z score log-sTfR		Z score log-ferritin	
	Non-adjusted	Adjusted*	Non-adjusted	Adjusted*
Premenopausal women				
High glucose †	1.35 (0.87-2.09)	1.23 (0.77-1.95)	0.89 (0.59-1.36)	1.01 (0.65-1.58)
Low HDL-C	1.18 (0.71-1.96)	1.14 (0.68-1.90)	0.95 (0.54-1.65)	0.96 (0.51-1.79)
High TG	1.35 (0.87-2.12)	1.29 (0.80-2.08)	1.51 (0.96-2.39)	1.51 (0.94-2.44)
High BP¶	1.35 (0.91-1.99)	1.29 (0.84-1.92)	0.95 (0.66-1.36)	0.96 (0.66-1.40)
High WC	1.07 (0.77-1.49)	1.01 (0.71-1.43)	0.79 (0.56-1.11)	0.79 (0.54-1.15)
MetS	1.32 (0.90-1.92)	1.21 (0.81-1.79)	1.13 (0.80-1.59)	1.24 (0.85-1.80)
Postmenopausal women				
High glucose †	1.08 (0.86-1.37)	1.009 (0.78-1.29)	1.23 (0.97-1.56)	1.20 (0.94-1.52)
Low HDL-C	1.22 (0.66-2.28)	1.45 (0.74-2.84)	0.76 (0.41-1.40)	0.81 (0.45-1.45)
High TG	0.99 (0.78-1.25)	0.97 (0.75-1.24)	1.26 (0.98-1.61)	1.28 (0.99-1.64)
High BP¶	1.13 (0.83-1.56)	0.94 (0.66-1.34)	1.14 (0.85-1.52)	1.08 (0.77-1.51)
High WC	1.10 (0.59-2.03)	1.00 (0.50-1.97)	1.10 (0.63-1.94)	0.98 (0.51-1.90)
MetS	0.99 (0.69-1.43)	0.83 (0.54-1.26)	**1.65 (1.17-2.31)**	**1.65 (1.11-2.46)**
Men				
High glucose †	1.20 (0.94-1.52)	1.10 (0.85-1.41)	**1.37 (1.07-1.75)**	**1.42 (1.10-1.84)**
Low HDL-C	0.93 (0.72-1.21)	0.94 (0.72-1.24)	**1.61 (1.21-2.15)**	**1.60 (1.19-2.15)**
High TG	1.03 (0.81-1.30)	1.07 (0.84-1.38)	**1.69 (1.29-2.21)**	**1.71 (1.29-2.26)**
High BP¶	1.13 (0.87-1.46)	0.97 (0.72-1.29)	1.15 (0.89-1.48)	1.21 (0.91-1.61)
High WC	1.30 (1.00-1.70)	1.16 (0.87-1.55)	**1.53 (1.18-1.98)**	**1.62 (1.22-2.15)**
MetS	1.05 (0.83-1.33)	0.95 (0.74-1.22)	**1.90 (1.44-2.50)**	**2.02 (1.51-2.70)**

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker) and alcohol consumption (no/yes). † Includes additionally individuals who reported current use of oral hypoglycemic medications or insulin regardless of fasting glucose values.¶ Includes additionally individuals who reported current use of antihypertensive medications regardless of blood pressure values. SD, standard deviation. TG, triglycerides. BP, blood pressure. WC, waist circumference. HDL-C, HDL cholesterol. MetS, metabolic syndrome. Significant associations are show in bold (P<0.05).

Table 25. Odds ratios (95% CI) for metabolic syndrome and its components per SD units of sTfR and ferritin levels in the whole sample

	Z score log-sTfR		Z score log-ferritin	
	Non-adjusted	Adjusted*	Non-adjusted	Adjusted*
High glucose †	1.11 (0.95-1.29)	1.08(0.90-1.28)	**1.48 (1.27-1.74)**	**1.27 (1.03-1.56)**
Low HDL-C	1.16 (0.98-1.36)	0.96 (0.74-1.23)	**0.46 (0.38-0.56)**	**1.34 (1.01-1.78)**
High TG	1.02 (0.88-1.19)	1.01 (0.85-1.21)	**1.71 (1.44-2.03)**	**1.62 (1.30-2.03)**
High BP¶	1.09 (0.93-1.28)	1.02 (0.84-1.23)	**1.52 (1.30-1.78)**	1.09 (0.85-1.38)
High WC	1.16 (0.97-1.39)	0.90 (0.71-1.15)	1.09 (0.91-1.29)	1.11 (0.80-1.56)
MetS	1.09 (0.94-1.27)	0.91 (0.75-1.09)	**1.41 (1.21-1.65)**	**1.93 (1.49-2.49)**

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes), BMI and sex/menopausal status (premenopausal women/ postmenopausal women/men). † Includes additionally individuals who reported current use of oral hypoglycemic medications or insulin regardless of fasting glucose values .¶ Includes additionally individuals who reported current use of antihypertensive medications regardless of blood pressure values. SD, standard deviation. TG, triglycerides. BP, blood pressure. WC, waist circumference. HDL-C, HDL cholesterol. MetS, metabolic syndrome. Significant associations are show in bold ($P<0.05$).

Table 26. Beta coefficients (95% CI) of relationship between log-sTfR and log-transformed values of variables related to MetS components

	log-sTfR	
Premenopausal women	Non-adjusted	Adjusted*
log-glucose	0.03 (-0.07 to 0.15)	0.01 (-0.09 to 0.12)
log- HDL-C	-0.006 (-0.15 to 0.14)	0.008 (-0.14 to 0.16)
log-TG	**0.32 (0.01 to 0.63)**	0.26 (-0.04 to 0.56)
log-SBP	0.07 (-0.01 to 0.17)	0.04 (-0.04 to 0.12)
log-DBP	0.01 (-0.07 to 0.11)	-0.002 (-0.08 to 0.08)
log- WC	0.06 (-0.02 to 0.15)	0.007 (-0.03 to 0.05)
Postmenopausal women		
log-glucose	0.06 (-0.12 to 0.24)	-0.04 (-0.22 to 0.14)
log- HDL-C	0.04 (-0.08 to 0.17)	0.07 (-0.06 to 0.21)
log-TG	0.01 (-0.37 to 0.40)	-0.14 (-0.53 to 0.23)
log-SBP	0.10 (-0.03 to 0.25)	-0.007 (-0.13 to 0.12)
log-DBP	-0.01 (-0.12 to 0.09)	-0.06 (-0.17 to 0.04)
log- WC	**0.15 (0.05 to 0.25)**	0.05 (-0.004 to 0.10)
Men		
log-glucose	**0.18 (0.002 to 0.37)**	0.09 (-0.09 to 0.27)
log- HDL-C	-0.03 (-0.18 to 0.11)	-0.02 (-0.18 to 0.12)
log-TG	0.33 (-0.14 to 0.81)	0.34 (-0.13 to 0.82)
log-SBP	0.10 (-0.03 to 0.25)	-0.01 (-0.14 to 0.11)
log-DBP	0.05 (-0.07 to 0.18)	0.01 (-0.11 to 0.14)
log- WC	**0.12 (0.02 to 0.22)**	0.01 (-0.02 to 0.06)

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes), and BMI. Multivariable adjusted analyses were performed on transformed values of skewed variables: logarithm of sTfR , ferritin , body mass index and fibrinogen values; square of age. . TG, triglycerides. SBP, systolic blood pressure. DBP, diastolic blood pressure. WC, waist circumference. HDL-C, HDL cholesterol. Significant associations are shown in bold (P<0.05).

Table 27. Odds ratios (95% CI) for metabolic syndrome and its components per SD units of iron markers in women of the study

	Z score log-sTfR		Z score log-ferritin	
	Non-adjusted	Adjusted*	Non-adjusted	Adjusted*
High glucose †	1.09 (0.90-1.33)	1.07 (0.85-1.36)	**1.53 (1.24-1.88)**	1.12 (0.88-1.43)
Low HDL-C	1.17 (0.81-1.69)	1.16 (0.84-1.61)	1.05 (0.71-1.55)	0.83 (0.52-1.31)
High TG	1.05 (0.86-1.28)	1.02 (0.80-1.30)	**1.66 (1.32-2.08)**	**1.41 (1.08-1.83)**
High BP¶	1.10 (0.90-1.34)	1.10 (0.85-1.42)	**1.74 (1.41-2.14)**	1.03 (0.77-1.36)
High WC	1.01 (0.78-1.31)	0.90 (0.67-1.20)	**1.53 (1.18-1.95)**	0.90 (0.57-1.42)
MetS	1.06 (0.86-1.30)	1.02 (0.78-1.30)	**2.22 (1.77-2.80)**	**1.55 (1.13-2.13)**

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes), BMI and menopause. † Includes additionally individuals who reported current use of oral hypoglycaemic medications or insulin regardless of fasting glucose values .¶ Includes additionally individuals who reported current use of antihypertensive medications regardless of blood pressure values. SD, standard deviation TG, triglycerides. BP, blood pressure.. WC, waist circumference. HDL-C, HDL cholesterol. MetS, metabolic syndrome. Significant associations are shown in bold (P<0.05).

Table 28. Adjusted* Beta coefficients (95% CI) for values of log-HOMA-IR and log-insulin by levels of iron markers in the study subjects

Independent variable	log-sTfR		log-ferritin	
Dependant variable	log-HOMA-IR	log-insulin	log-HOMA-IR	log-insulin
In				
Premenopausal women	0.07 (-0.12 to 0.27)	0.15 (-0.27 to 0.58)	0.05 (-0.05 to 0.16)	0.09 (-0.14 to 0.33)
Postmenopausal women	**0.34 (0.05 to 0.63)**	**0.83 (0.23 to 1.43)**	0.01 (-0.09 to 0.11)	0.01 (-0.19 to 0.23)
Men	**0.44 (0.14 to 0.75)**	**0.98 (0.34 to 1.63)**	0.01 (-0.09 to 0.11)	-0.03 (-0.26 to 0.18)
All**	**0.24 (0.09 to 0.39)**	**0.54 (0.23 to 0.86)**	0.04 (-0.01 to 0.10)	0.06 (-0.06 to 0.19)

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes), treatment with insulin and/or hypoglycaemic drugs (yes/no), and BMI. ** Additionally adjusted for sex/menopausal status (premenopausal women [reference] / postmenopausal women/men). Multivariable adjusted analyses were performed on transformed values of skewed variables: logarithm of sTfR, ferritin, HOMA-IR values, body mass index and fibrinogen values; square of age. Significant associations are shown in bold (P<0.05).

Table 29. Beta coefficients (95% CI) for values of glycosylated haemoglobin (HbA1C) by levels of iron markers in the study subjects

	log-sTfR		log-ferritin	
	Non-adjusted	Adjusted*	Non-adjusted	Adjusted*
Premenopausal women	-0.06 (-0.14 to 0.01)	-0.06 (-0.14 to 0.01)	0.02 (-0.01 to 0.07)	0.02 (-0.01 to 0.07)
Postmenopausal women	0.02 (-0.13 to 0.17)	-0.04 (-0.17 to 0.08)	-0.007 (-0.06 to 0.04)	-0.009 (-0.05 to 0.03)
Men	**0.18 (0.03 to 0.33)**	0.03 (-0.09 to 0.16)	0.02 (-0.02 to 0.07)	0.001 (-0.04 to 0.04)
All**	0.01 (-0.05 to 0.09)	-0.03 (-0.10 to 0.02)	**0.05 (0.01 to 0.08)**	0.005 (-0.02 to 0.03)

*Age, fibrinogen levels, smoking status (yes/no/ex-smoker), alcohol consumption (no/yes), treatment with insulin and/or hypoglycaemic drugs (yes/no), and BMI. * Additionally adjusted for sex/menopausal status (premenopausal women [reference] / postmenopausal women/men). Multivariable adjusted analyses were performed on transformed values of skewed variables: logarithm of sTfR , ferritin , body mass index and fibrinogen values; square of age and square root(sqrt) of glycosylated haemoglobin. Significant associations are shown in bold (P<0.05).

Table 30. Differences between included and excluded subjects of the SHeS 1995 and 1998 (Longitudinal analysis Chapter 5) [weighted values]

	Included (*n*=6497)*	Excluded (*n*=10775)*	*n* for variables in excluded	P value
Variables				
Age	41(31-52)	38(26-52)	*n*=10775	<0.001
Pre-W/Post-W/ Men (%)	34.5/20.7/44.9	32.6/21.7/45.7	*n*=10266	0.397
Ferritin (µg/L)	61(32-108)	61(34-107)	*n*=5503	0.312
Fibrinogen (g/L)	2.9(2.4-3.5)	3.0(2.4-3.6)	*n*=4770	0.042
GGT (IU/L)	20(14-32)	21(15-32)	*n*=5680	0.165
Smoking status (%)			*n*=10443	
Never smoker	39	40.3		0.009
Ex-regular or Ex-occasional smoker	26.3	24.2		
Current smoker	34.7	35.5		
Alcohol consumption Categories of rating units/week Prevalence (%)			*n*=10371	
Never drank	4.2	5.9		<0.001
Ex-drinker	2.8	4.1		
Trivial drinker/Non-zero but under 1	10.3	12.2		
1-20	63.4	59.1		
≥ 21	19.3	18.8		
BMI (Kg/mts^2)	25.7(23.1-28.6)	25.4(22.5-28.8)	*n*=9208	<0.001
HDL-cholesterol (mmol/L)	1.4(1.2-1.7)	1.4(1.1-1.6)	*n*=5433	<0.001
Total cholesterol (mmol/L)	5.4(4.7-6.2)	5.4(4.6-6.2)	*n*=5559	
Systolic blood pressure (mmHg)	70(63-79)	70(62-78)	*n*=6081	<0.001
Diastolic blood pressure (mmHg)	126(117-137)	127(117-138)	*n*=6081	0.025
Incident diabetes (%)**	4.9	8.2	*n*=9171	<0.001
Incident coronary *heart dise*ase (%)**	5.3	7.9	*n*=9171	<0.001
Incident cerebrovascular disease (%)**	2.4	3.3	*n*=9171	<0.001
Survey 1995/1998 (%)	50.1/49.9	43.9/56.1	*n*=10775	<0.001

*Samples sizes are based on unweighted values. Data are median (interquartile range) or %. Comparison between groups by Mann-Whitney U and χ^2 test. Pre-MW, premenopausal women. Post-MW, postmenopausal women. BMI, body mass index. GGT, gamma-glutamyl transpeptidase.

Table 31. Baseline characteristics of the SHeS 95-98 participants and incidence of outcome diseases by sex-and menopausal stats-specific quartiles of ferritin level in the study cohort (n=6497) [unweighted values]

		Ferritin (µg/L)				
	All	**Q1**	**Q2**	**Q3**	**Q4**	**P for trend**
*Ferritin levels range by sex/menopausal status (*µg/L)						
Premenopausal women (*n=2241*)	2.0-950	2.0-18	19-30	31-47	48-950	
Postmenopausal women (*n=1345*)	3.0-1000	3.0-34	35-58	58-91	92-1000	
Men (*n=2924*)	3.0-2251	3.0-61	62-96	97-151	152-2251	
Variables						
Age	43(32-54)	41(32-52)	41(31-54)	42(32-54)	46(35-57)	<0.001
Pre-W/Post-W/ Men (%)	34.4/20.7/44.9	33.8/20.6/45.6	34.5/20.9/44.6	35.3/20.3/44.4	34.2/20.9/45	0.669
Ferritin (µg/L)	59(32-105)	23(13-41)	51(27-76)	81(41.2-114)	156(82-209)	<0.001
Fibrinogen (g/L)	3.0(2.5-3.6)	2.8(2.4-3.5)	2.9(2.5-3.5)	3.0(2.5-3.7)	3.1(2.6-3.7)	<0.001
GGT (U/L)	20(14-31)	17(13-26)	18(14-27)	21(15-32)	26(18-44)	<0.001
Smoking *n (%)*						
Never smoker	2474(38)	714(44.1)	640(39.7)	584(35.4)	536(32.9)	<0.001
Ex-regular or Ex-occasional smoker	1728(26.5)	408(25.2)	434(26.9)	440(26.7)	446(27.4)	
Current smoker	2308(35.5)	497(30.7)	539(33.4)	624(37.9)	648(39.8)	
Alcohol consumption Categories of rating units/week *n (%)*						
Never drank	289(4.4)	93(5.7)	83(5.1)	63(3.8)	50(3.1)	<0.001
Ex-drinker	211(3.2)	61(3.8)	55(3.4)	55(3.3)	40(2.5)	
Trivial drinker/Non-zero but under 1	757(11.6)	231(14.3)	208(12.9)	166(10.1)	152(9.3)	
1-20	4102(63)	1023(63.2)	1009(62.6)	1065(64.6)	1005(61.7)	

		Ferritin (µg/L)				
	All	**Q1**	**Q2**	**Q3**	**Q4**	**P for trend**
≥ 21	1151(17.7)	211(13)	258(16)	299(18.1)	383(23.5)	
BMI (Kg/mts^2)	25.7(23.1-28.7)	25(22.7-27.8)	25.3(22.9-28.2)	25.8(23.3-28.7)	26.5(23.9-29.9)	<0.001
HDL-cholesterol (mmol/L)	1.4(1.2-1.7)	1.4(1.2-1.7)	1.4(1.2-1.7)	1.4(1.2-1.7)	1.4(1.1-1.7)	0.049
Total cholesterol (mmol/L)	5.4(4.8-6.3)	5.3(4.6-6.1)	5.4(4.7-6.3)	5.5(4.8-6.3)	5.7(4.9-6.5)	<0.001
Systolic blood pressure (mmHg)	126.5(117-138)	126(117-1365)	125(116-137)	126(117-138)	129.5(119-142)	<0.001
Diastolic blood pressure (mmHg)	71(64-79.5)	70(63-78)	70(63-78.5)	71(64-79)	73(65-81)	<0.001
Development of diabetes (%)	340(5.2)	56(3.5)	67(4.2)	75(4.6)	142(8.7)	<0.001
Development of coronary heart disease n (%)	375(5.8)	81(5.0)	81(5.0)	90(5.5)	123(7.5)	0.002
Development of cerebrovascular disease n (%)	183(2.8)	38(2.3)	38(2.4)	47(2.9)	60(3.7)	0.014
Survey 1995/1998 n (%)	49.8/50.2	44/56	50.1/49.9	52.9/47.1	52/48	<0.001
Data are median (interquartile range) or %. Comparison between groups by Mann-Whitney U and χ^2 test. Pre-MW, premenopausal women. Post-MW, postmenopausal women. BMI, body mass index. GGT, gamma-glutamyl transpeptidase.						

Table 32. HRs and 95% CI for the incidence of diabetes and cardiovascular diseases by serum ferritin levels [unweighted Cox regressions]

	Type 2 diabetes			Coronary heart disease			Cerebrovascular disease		
	Unadjusted	**Age- and sex/menopausal status-adjusted**	**Fully adjusted**	**Unadjusted**	**Age- and sex/menopausal status-adjusted**	**Fully adjusted**	**Unadjusted**	**Age- and sex/menopausal status-adjusted**	**Fully adjusted**
Z score of log-ferritin	**1.49 (1.34-1.66) P<0.001**	**1.41 (1.27-1.57) P<0.001**	**1.18 (1.05-1.31) P=0.003**	**1.17 (1.05-1.30) P=0.002**	1.09 (0.99-1.21) P=0.071	1.02 (091-1.13) P=0.707	**1.19 (1.03-1.39) P=0.016**	1.12 (0.97-1.29) P=0.118)	1.04 (0.90-1.21) P=0.548
Ferritin									
Quartile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Quartile 2	1.18 (0.83-1.69)	1.15 (0.80-1.64)	1.16 (0.81-1.66)	0.99 (0.73-1.35)	0.96 (0.70-1.30)	0.90 (0.66-1.23)	0.98 (0.63-1.55)	0.94 (0.60-1.48)	0.93 (0.59-1.46)
Quartile 3	1.30 (0.91-1.83)	1.24 (0.87-1.75)	1.07 (0.75-1.53)	1.07 (0.79-1.45)	1.01 (0.75-1.37)	0.88 (0.65-1.20)	1.19 (0.77-1.82)	1.12 (0.73-1.72)	1.01 (0.66-1.5 7)
Quartile 4	**2.60 (1.91-3.55)**	**2.25 (1.65-3.06)**	**1.54 (1.11-2.13)**	**1.53 (1.15-2.02)**	**1.26 (0.95-1.67)**	1.04 (0.78-1.41)	**1.57 (1.05-2.37)**	1.30 (0.86-1.95)	1.09 (0.71-1.68)
P for trend	**P<0.001**	**P<0.001**	**P=0.009**	**P=0.002**	**P=0.070**	P=0.708	**P=0.015**	P=0.125	P=0.562

*Adjusted for age, sex/menopausal status, fibrinogen levels, GGT levels, alcohol intake, smoking, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, body mass index and year of survey

Table 33. Differences between included and excluded subjects in the ET2DS

	Included (*n*=821)	Excluded (*n*=118)	*n* for variables in excluded	P value
Sex Male/Female n	422(48.6)/399(51.4)	66(55.9)/52(44.1)	*n*=118	0.205
Variables				
Age (years)	68.9(65.3-72.3)	69.4(65.3-73.4)	*n*=118	0.332
Duration of diabetes (years)	7.0(4.0-12)	7.0(5.0-13)	*n*=110	0.616
Ferritin (µg/L)	73(32.5-141)	48(24-124)	*n*=55	0.127
Glucose mmol/L	6.4(5.4-7.9)	6.5(5.2-7.8)	*n*=103	0.819
HbA1C (%)	7.0(6.4-7.7)	7.4(6.7-8.2)	*n*=118	<0.001
Fibrinogen (g/L)	3.5(3.1-4.0)	3.4(3.1-4.0)	*n*=118	0.134
CRP(mg/L)	1.7(0.8-4.0)	1.8(0.6-4.4)	*n*=99	0.737
GGT(U/L)	17(10-31)	13(8.0-22.7)	*n*=112	0.004
ALT(U/L)	31(26-31)	29(23-35)	*n*=112	0.007
AST(U/L)	29(24-35)	26(21.2-31)	*n*=112	<0.001
Smoking [ever] n(%)	490(59.7)	72(61)	*n*=118	0.432
Alcohol consumption [ever] n(%)	673(82)	99(85.3)	*n*=116	0.226
BMI (Kg/mts^2)	30.6(27.4-34.4)	30.2(26.4-34.4)	*n*=117	0.446
SBP mmHg	132(112-142)	134(122-142)	*n*=116	0.735
DBP mmHg	68(62-76)	68(60.5-76)	*n*=116	0.541
HDL-cholesterol (mmol/L)	1.2(1.1-1.5)	1.3(1.1-1.6)	*n*=110	0.055
Total cholesterol (mmol/L)	4.2(3.7-4.7)	4.3(3.8-5.0)	*n*=110	0.261
eGFR, mL/min/1.73 m^2	60(60-71)	66(59.7-82.2)	*n*=106	0.012
Oral hypoglycaemic drugs n(%)	607(73.9)	92(78)	*n*=118	0.206
Antihypertensive drugs n(%)	705(85.9)	101(85.6)	*n*=118	0.514
Lipid-lowering drugs n(%)	691(84.2)	102(86.4)	*n*=118	0.314
Insulin therapy n(%)	123(15)	25(21.2)	*n*=118	0.059
Liver disease n(%)	12(1.5)	3(2.5)	*n*=118	0.289
Any cardiovascular event *at baseline n(%)*	285(34.7)	48(40.7)	*n*=118	0.123
Outcomes after follow-up				
Development of CHD	78(9.5)	13(16.9)	*n*=118	0.351
Development of CEVD	54(6.6)	9(7.6)	*n*=118	0.394
Development of CVD	123(15)	20(16.9)	*n*=118	0.331

Data are median (interquartile range) or *n* (%). Differences by groups by Mann-Whitney U test (continuous variables) and χ^2 tests (categorical variables). GGT, gamma-glutamyl transpeptidase, ALT, Alanine aminotransferase. AST, Aspartate aminotransferase. CRP, C reactive protein. HDL-C, HDL cholesterol. BMI, body mass index. eGFR, estimate glomerular filtration rate. HbA1C, glycosylated haemoglobin. SBP, systolic blood pressure. DBP, diastolic blood pressure.

Table 34. Differences between included and excluded subjects in the SIDIAP study

	Included (Those with no missing values)	**Excluded**	***n* for variables in the excluded**	**P value**
	(*n*=38617)	(n=279403)		
Sex Male/Female n	16423/22194	154796/124607	n=279403	<0.001
Variables				
Age (years)	73(64-80)	69(60-78)	n=279403	<0.001
Duration of diabetes	7(4-10)	6(3-9)	n=279403	<0.001
Ferritin (µg/L)	62(26-143)	69.7(28.8-163)	n=23385	<0.001
Triglycerides (mmol/L)	1.4(1.0-1.9)	1.4(1.0-2.0)	n=209205	<0.001
HbA1C (%)	6.9(6.3-7.8)	6.9(6.3-7.8)	n=212353	0.216
White blood cells count x 1000/µL	7.2(6.0-8.5)	7.2(6.1-8.6)	n=202756	<0.001
Smoking [ever] n(%)	11487(29.7)	101335(36.3)	n=271566	<0.001
Alcohol consumption [ever] n(%)	7882(20.4)	67462(24.1)	n=279403	<0.001
BMI (Kg/mts^2)	29.3(26.4-32.9)	29.4(26.6-32.8)	n=199640	<0.001
SBP mmHg	134(125-141)	134(126-140)	n=227881	0.065
DBP mmHg	74(67-80)	76(70-81)	n=227881	<0.001
HDL-cholesterol (mmol/L)	1.2(1.0-1.4)	1.2(1.0-1.4)	n=198687	0.624
Total cholesterol (mmol/L)	4.6(4.0-5.2)	4.8(4.1-5.4)	n=221018	<0.001
eGFR, mL/min/1.73 m^2	60(54-60)	60(60-60)	n=217425	<0.001
Oral hypoglycaemic drugs n(%)	29331(76.0)	195786(70.1)	n=279403	<0.001
Antihypertensive drugs n(%)	31064(80.4)	195551(70)	n=279403	<0.001
Lipid-lowering drugs n(%)	23770(61.6)	152157(54.5)	n=279403	<0.001
Insulin therapy n(%)	8815(22.8)	48831(17.5)	n=279403	<0.001
Cardiovascular diseases n(%)				
CHD	6143(15.9)	33692(12.1)	n=279403	<0.001
CEVD	2812(7.3)	16042(5.7)	n=279403	<0.001
CVD (any cardiovascular event)	8286(21.5)	46478(16.6)	n=279403	<0.001

Data are median (interquartile range) or *n* (%). Differences by groups by Mann-Whitney U test (continuous variables) and χ^2 tests (categorical variables). HDL-C, HDL cholesterol. BMI, body mass index. eGFR, estimate glomerular filtration rate. HbA1C, glycosylated haemoglobin. SBP, systolic blood pressure. DBP, diastolic blood pressure.

Table 35. Clinical and biochemical variables measured in the two studies of people with Type 2 diabetes

	ET2DS (baseline)	SIDIAP (Cross-sectional)
	(*n*=821)	(*n*=38617)
Sex Male/Female n	422/399	16423/22194
*Ferritin levels range by sex (*µg/L)		
Men	7-1095	1.0-3507
Women	5-605	1.0-3682
Variables		
Age (years)	68 (65-73)	73(64-80)
Duration of diabetes	7(4-12)	7(4-10)
Ferritin (µg/L)	73(32.5-141)	62(26-143)
HbA1C (%)	7.1(6.4-7.7)	6.9(6.3-7.8)
Smoking [ever] n(%)	490(59.7)	11487(29.7)
Alcohol consumption [ever] n(%)	673(82)	7882(20.4)
BMI (Kg/mts^2)	30.6(27.4-34.4)	29.3(26.4-32.9)
SBP mmHg	132(122-142)	134(125-141)
DBP mmHg	68(62-76)	74(67-80)
HDL-cholesterol (mmol/L)	1.2(1.1-1.5)	1.2(1.0-1.4)
Total cholesterol (mmol/L)	42(3.7-4.7)	4.6(4.0-5.2)
eGFR, mL/min/1.73 m^2	60(60-71)	60(54-60)
Oral hypoglycaemic drugs n(%)	607(73.9)	29331(76.0)
Antihypertensive drugs n(%)	705(85.9)	31064(80.4)
Lipid-lowering drugs n(%)	691(84.2)	23770(61.6)
Insulin therapy n(%)	123(15)	8815(22.8)
CHD (%)	250(30.5)	6143(15.9)
CEVD (%)	71(8.6)	2812(7.3)
CVD (any cardiovascular event) (%)	285(34.7)	8286(21.5)

Data are median (interquartile range) or *n* (%). HDL-C, HDL cholesterol. BMI, body mass index. eGFR, estimate glomerular filtration rate. HbA1C, glycosylated haemoglobin. SBP, systolic blood pressure. DBP, diastolic blood pressure. CHD, coronary heart disease. CEVD, cerevrovascular disease. CVD, cardiovascular disease.

Table 36 .Odds ratios and 95% CI for prevalent cardiovascular events by ferritin levels and sex in the SIDIAP study

	Men			Women		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
CHD						
Ferritin (Z score of log-ferritin)	**0.64 (0.60-0.67) P<0.001**	**0.67 (0.63-0.71) P<0.001**	**0.74 (0.70-0.79) P<0.001**	**0.93 (0.89-0.98) P<0.001**	**0.92 (0.87-0.96) P<0.001**	**0.91 (0.87-0.96) P<0.001**
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.000 (reference)	1.00 (reference)	1.00(reference)	1.00(reference)
Ferritin Quintile 2	**0.82 (0.73-0.91)**	**0.81 (0.73-0.90)**	**0.82 (0.73-0.93)**	1.11 (0.98-1.26)	1.02 (0.89-1.15)	1.02 (0.89-1.17)
Ferritin Quintile 3	**0.70 (0.62-0.78)**	**0.72 (0.64-0.80)**	**0.78 (0.69-0.88)**	1.03 (0.90-1.17)	0.93 (0.81-1.06)	0.93 (0.81-1.07)
Ferritin Quintile 4	**0.49 (0.43-0.55)**	**0.52 (0.46-0.59)**	**0.60 (0.53-0.69)**	**0.86 (0.76-0.99)**	**0.79 (0.69-0.91)**	**0.83 (0.72-0.95)**
Ferritin Quintile 5	**0.36 (0.32-0.41)**	**0.40 (0.35-0.46)**	**0.50 (0.44-0.58)**	**0.81 (0.71-0.93)**	**0.74 (0.65-0.85)**	**0.76 (0.66-0.88)**
P for trend	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
CEVD						
Ferritin (Z score of log-ferritin)	**0.82 (0.77-0.88) P<0.001**	**0.88 (0.82-0.94) P<0.001**	**0.93 (0.87-0.99) P=0.005**	0.95 (0.89-1.01) P=0.133	0.93 (0.87-1.00) P=0.056	**0.91 (0.85-0.98) P=0.013**
Ferritin Quintile 1	1.00 (reference)	1.00(reference)	1.00(reference)	1.00 (reference)	1.00(reference)	1.00(reference)
Ferritin Quintile 2	**0.82 (0.70-0.95)**	**0.80 (0.68-0.94)**	**0.80 (0.68-0.94)**	1.07 ((0.90-1.26)	0.97 (0.82-1.15)	0.97 (0.81-1.15)
Ferritin Quintile 3	**0.72 (0.61-0.84)**	**0.74 (0.63-0.88)**	**0.76 (0.65-0.90)**	1.00 (0.84-1.18)	0.90 (0.76-1.07)	0.89 0.74-1.05)
Ferritin Quintile 4	**0.69 (0.58-0.81)**	**0.76 (0.65-0.90)**	**0.82 (0.69-0.97)**	**0.81 (0.68-0.97)**	**0.74 (0.62-0.89)**	**0.74 (0.62-0.89)**
Ferritin Quintile 5	**0.55 (0.46-0.65)**	**0.64 (0.54-0.76)**	**0.73 (0.61-0.88)**	**0.73 (0.60-0.88)**	**0.67 (0.55-0.80)**	**0.64 (0.52-0.77)**
P for trend	<0.001	<0.001	0.003	<0.001	<0.001	<0.001
CVD						
Ferritin (Z score of log-ferritin)	**0.67 (0.64-0.71) P<0.001**	**0.72 (0.68-0.75) P<0.001**	**0.79 (0.75-0.83) P<0.001**	**0.94 (0.90-0.98) P=0.004**	**0.92 (0.88-0.96) P<0.001**	**0.91 (0.87-0.95) P<0.001**
Ferritin Quintile 1	1.00 (reference)	1.00(reference)	1.00(reference)	1.00 (reference)	1.00(reference)	1.00(reference)
Ferritin Quintile 2	**0.79 (0.72-0.88)**	**0.78 (0.70-0.87)**	**0.79 (0.71-0.89)**	1.08 (0.97-1.20)	0.98 (0.88-1.10)	0.99 (0.88-1.11)
Ferritin Quintile 3	**0.66 (0.59-0.73)**	**0.68 (0.61-0.75)**	**0.72 (0.64-0.81)**	1.02 (0.91-1.14)	0.91 (0.81-1.02)	0.91 (0.81-1.02)
Ferritin Quintile 4	**0.51 (0.46-0.56)**	**0.55 (0.50-0.62)**	**0.63 (0.56-0.71)**	**0.83 (0.74-0.93)**	**0.75 (0.67-0.85)**	**0.78 (0.69-0.88)**
Ferritin Quintile 5	**0.37 (0.33-0.41)**	**0.42 (0.37-0.47)**	**0.51 (0.45-0.58)**	**0.77 (0.68-0.86)**	**0.69 (0.61-0.78)**	**0.70 (0.61-0.79)**
P for trend	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

CHD, coronary heart disease. CEVD cerebrovascular disease. CVD, cardiovascular disease. Model 1: unadjusted. Model 2: adjusted for age. Model 3: adjusted for adjusted for age, duration of diabetes, use of specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking status, alcohol consumption, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, HDL-cholesterol, total cholesterol, white blood cells count, triglycerides level, and estimated glomerular filtration rate (eGFR). Significant associations are shown in bold (P<0.05).

Table 37. Odds ratios and 95% CI for prevalent cardiovascular events by ferritin levels and sex in the ET2DS (n=821)

	Men			Women		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
CHD						
Ferritin (Z score of log-ferritin)	0.82 (0.66-1.01) P=0.066	0.83 (0.67-1.02) P=0.083	0.83 (0.64-1.07) P=0.153	0.88 (0.69-1.12) P=0.312	0.87 (0.68-1.11) P=0.280	0.78 (0.57-1.05) P=0.111
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	1.02 (0.55-1.90)	1.10 (0.59-2.06)	1.36 (0.68-2.73)	0.41 (0.19-0.89)	0.42 (0.19-0.90)	0.38 (0.15-0.91)
Ferritin Quintile 3	0.64 (0.34-1.22)	0.68 (0.36-1.30)	0.65 (0.31-1.33)	0.87 (0.44-1.71)	0.88 (0.45-1.73)	0.63 (0.28-1.40)
Ferritin Quintile 4	0.90 (0.48-1.68)	0.96 (0.51-1.80)	0.94 (0.46-1.93)	0.57 (0.27-1.17)	0.57 (0.27-1.17)	0.52 (0.22-1.25)
Ferritin Quintile 5	0.65 (0.34-1.24)	0.69 (0.36-1.32)	0.83 (0.39-1.79)	0.61 (0.30-1.24)	0.59 (0.29-1.21)	**0.38 (0.15-0.97)**
P for trend	0.182	0.232	0.396	0.353	0.308	0.098
CEVD						
Ferritin (Z score of log-ferritin)	0.94 (0.68-1.30) P=0.737	0.95 (0.69-1.30) P=0.754	1.03 (0.71-1.51) P=0.846	0.83 (0.62-1.11) P=0.226	0.84 (0.62-1.12) P=0.248	0.80 (0.56-1.15) P=0.242
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	0.66 (0.24-1.83)	0.67 (0.24-1.87)	0.77 (0.26-2.24)	1.64 (0.51-5.2)	1.61 (0.50-5.19)	1.33 (0.35-4.97)
Ferritin Quintile 3	0.66 (0.24-1.83)	0.67 (0.24-1.87)	0.58 (0.19-1.75)	0.94 (0.26-3.41)	0.94 (0.26-3.38)	0.76 (0.18-3.19)
Ferritin Quintile 4	1.60 (0.67-3.81)	1.63 (0.68-3.89)	1.92 (0.72-5.10)	0.76 (0.19-2.97)	0.76 (0.19-2.96)	0.68 (0.15-2.97)
Ferritin Quintile 5	0.67 (0.24-1.86)	0.68 (0.24-1.89)	0.89 (0.29-2.77)	0.56 (0.13-2.46)	0.58 (0.13-2.56)	0.44 (0.07-2.45)
P for trend	0.816	0.793	0.531	0.226	0.248	0.242
CVD						
Ferritin (Z score of log-ferritin)	0.83 (0.68-1.02) P=0.085	0.84 (0.68-1.03) P=0.101	0.83 (0.64-1.06) P=0.141	0.86 (0.68-1.08) P=0.206	0.85 (0.68-1.07) P=0.183	0.74 (0.55-1.00) P=0.051
Ferritin Quintile 1	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile 2	1.02 (0.56-1.87)	1.01 (0.58-1.98)	1.29 (0.65-2.55)	0.52 (0.26-1.06)	0.53 (0.26-1.07)	0.48 (0.21-108)
Ferritin Quintile 3	0.50 (0.27-0.95)	0.53 (0.28-1.00)	0.45 (0.22-0.93)	0.82 (0.43-1.59)	0.83 (0.43-1.60)	0.57 (0.26-1.23)
Ferritin Quintile 4	0.86 (0.47-1.59)	0.90 (0.48-1.67)	0.84 (0.41-1.71)	0.55 (0.27-1.11)	0.55 (0.27-1.11)	0.47 (0.20-1.07)
Ferritin Quintile 5	0.71 (0.38-1.31)	0.73 (0.39-1.37)	0.84 (0.40-1.76)	0.63 (0.32-1.25)	0.61 (0.31-1.22)	**0.40 (0.16-0.96)**
P for trend	0.218	0.263	0.335	0.255	0.220	0.052

CI, confidence interval. CHD, coronary heart disease. CEVD, cerebrovascular disease. CVD, cardiovascular disease. Model 1: unadjusted. Model 2: adjusted for age. Model 3: adjusted for adjusted for age, duration of diabetes, use specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking status, alcohol consumption, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, fasting glucose, HDL-cholesterol, total cholesterol, C reactive protein (CRP), fibrinogen, transferases levels, liver disease and estimated glomerular filtration rate (eGFR) at baseline. Significant associations are shown in bold.

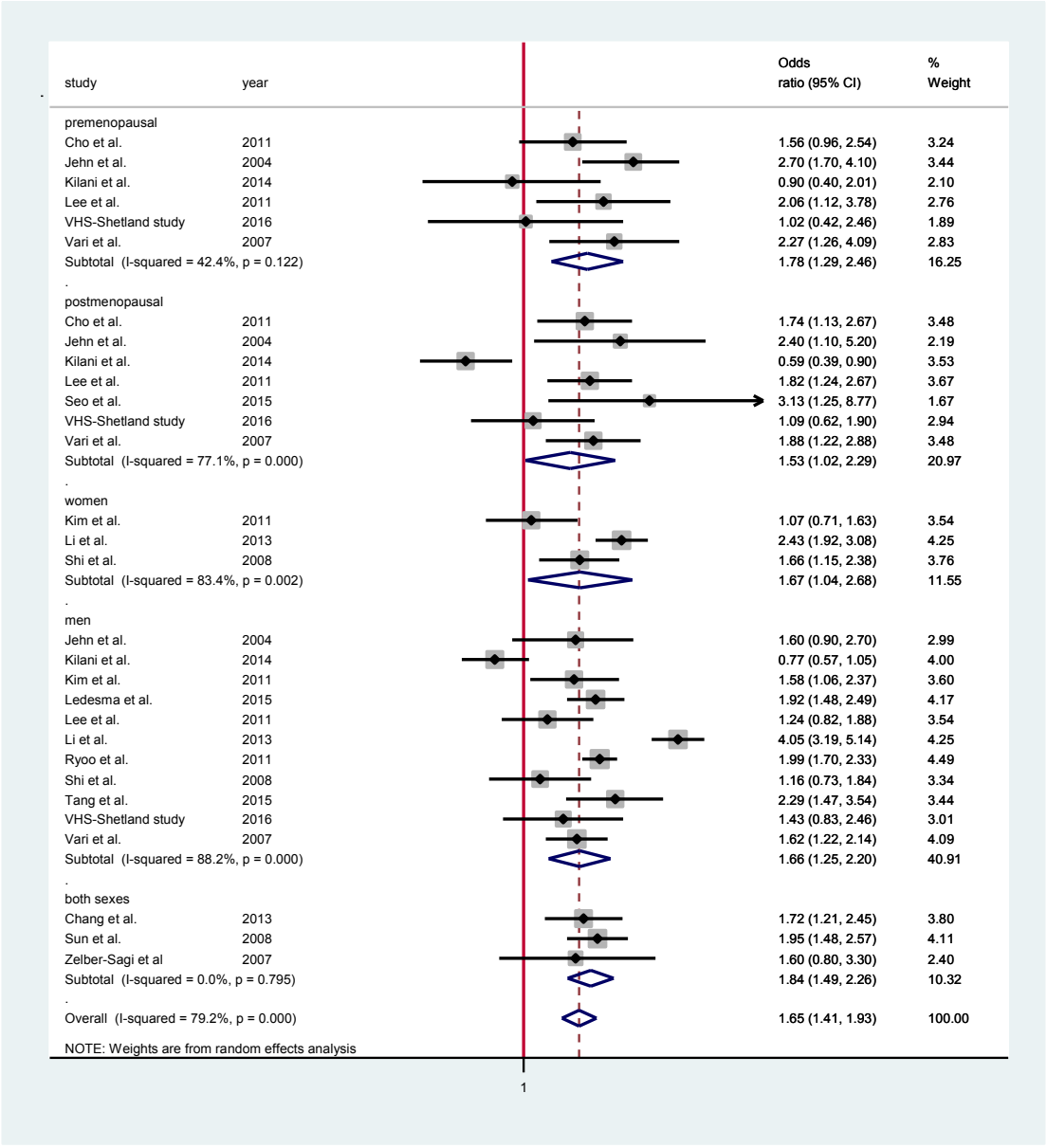
Table 38. HRs and 95% CI for incident cardiovascular events by ferritin levels* at baseline in the ET2DS (n=536)

	Model 1	Model 2	Model 3
CHD			
Ferritin (Z score of log-ferritin)	0.75(0.54-1.04) P=0.092	0.76(0.54-1.06) P=0.115	0.82(0.55-1.23) P=0.360
Ferritin Quintile 1 (*n*=95)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile2 (*n*=106)	0.28(0.09-0.90)	0.29(0.09-0.93)	0.36(0.10-1.18)
Ferritin Quintile 3 (*n*=115)	0.69(0.29-1.64)	0.72(0.30-1.71)	0.76(0.30-1.90)
Ferritin Quintile 4 (*n*=109)	0.72(0.30-1.71)	0.75(0.31-1.77)	0.90(0.34-2.35)
Ferritin Quintile 5 (*n*=111)	**0.20(0.05-0.74)**	**0.21(0.05-0.76)**	0.28(0.07-1.14)
P for trend	0.110	0.117	0.341
CEVD			
Ferritin (Z score of log-ferritin)	0.87(0.58-1.29) P=0.494	0.81(0.54-1.22) P=0.326	0.88(0.54-1.43) P=0.616
Ferritin Quintile 1 (*n*=95)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile2 (*n*=106)	0.35(0.09-1.35)	0.41(0.10-1.60)	0.60(0.12-2.88)
Ferritin Quintile 3 (*n*=115)	0.77(0.27-2.21)	0.87(0.30-2.50)	1.12(0.29-4.18)
Ferritin Quintile 4 (*n*=109)	0.34(0.08-1.32)	0.36(0.09-1.43)	0.47(0.09-2.30)
Ferritin Quintile 5 (*n*=111)	0.56(0.17-1.78)	0.59(0.19-1.89)	0.70(0.16-2.95)
P for trend	0.366	0.373	0.545
CVD			
Ferritin (Z score of log-ferritin)	0.79(0.61-1.03) P=0.087	0.78 (0.60-1.02) P=0.077	0.80(0.59-1.09) P=0.163
Ferritin Quintile 1 (*n*=95)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Ferritin Quintile2 (*n*=106)	0.32(0.13-0.78)	0.35(0.14-0.84)	0.44(0.17-1.09)
Ferritin Quintile 3 (*n*=115)	0.73(0.36-1.44)	0.78(0.39-1.55)	0.83(0.40-1.72)
Ferritin Quintile 4 (*n*=109)	0.57(0.27-1.19)	0.60(0.28-1.26)	0.64(0.28-1.45)
Ferritin Quintile 5 (*n*=111)	**0.36(0.15-0.84)**	**0.37(0.16-0.86)**	0.42(0.16-1.08)
P for trend	0.085	0.092	0.166

HR, hazard ratio. CI, confidence interval. CHD, coronary heart disease. CEVD, cerebrovascular disease. CVD, cardiovascular disease. Model 1: unadjusted. Model 2: adjusted for age and sex. Model 3: adjusted for adjusted for age, sex, duration of diabetes, use specific anti-hyperglycaemic agents, treatment with insulin, lipid-lowering drugs, blood pressure-lowering drugs, smoking status, alcohol consumption, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA1c, fasting glucose, HDL-cholesterol, total cholesterol, C reactive protein (CRP), fibrinogen, transferases levels, liver disease and estimated glomerular filtration rate (eGFR) at baseline. Values of transaminases levels, glucose, cholesterol, CRP and duration of diabetes were log-transformed for the analysis. Significant associations are shown in bold (P<0.05). *Cut-offs for quintiles from the whole sample (n=821) were maintained.

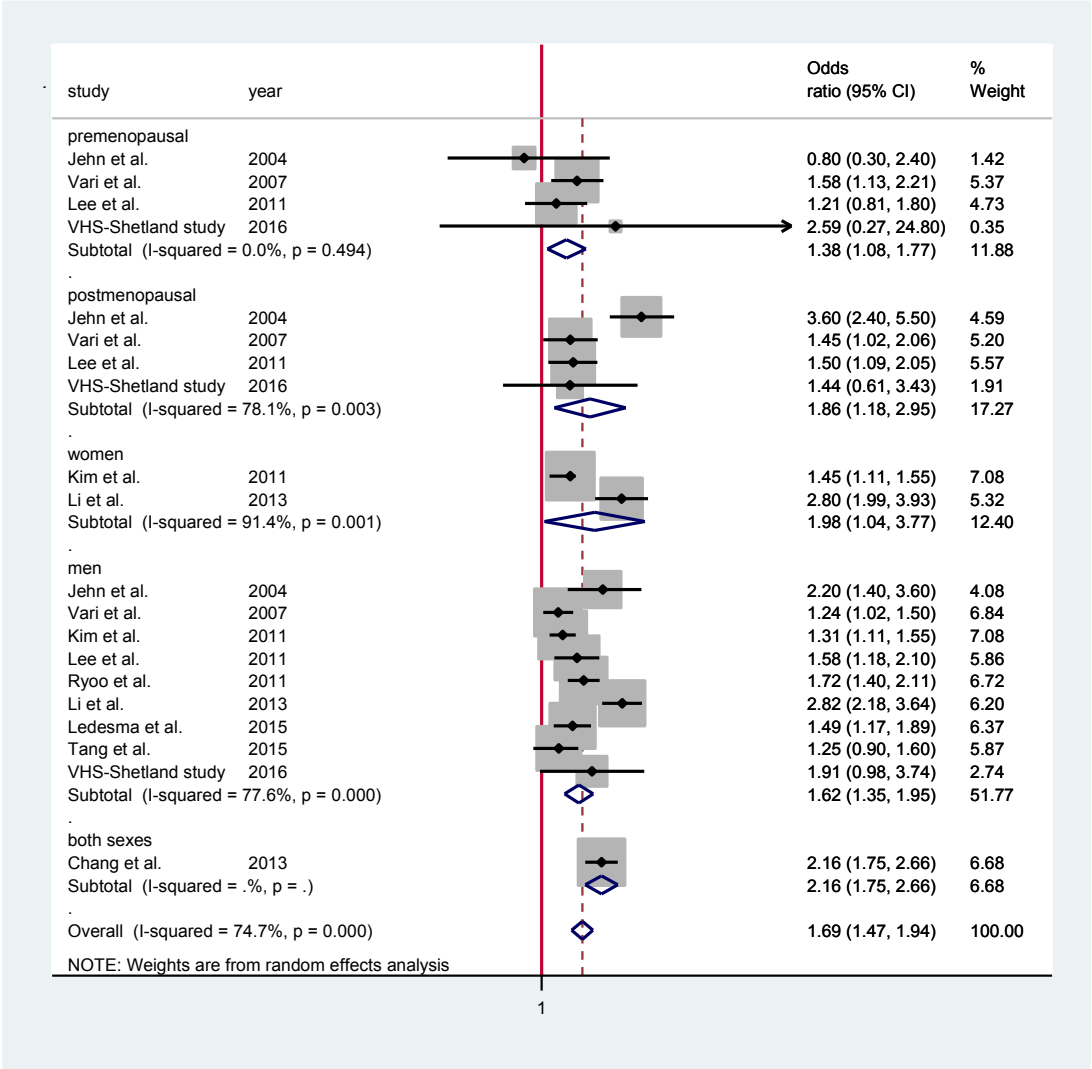
Appendix figures

Figure 1. Forest plot describing the association between ferritin and metabolic syndrome in observational studies (cross-sectional only [Meta-analysis 2].



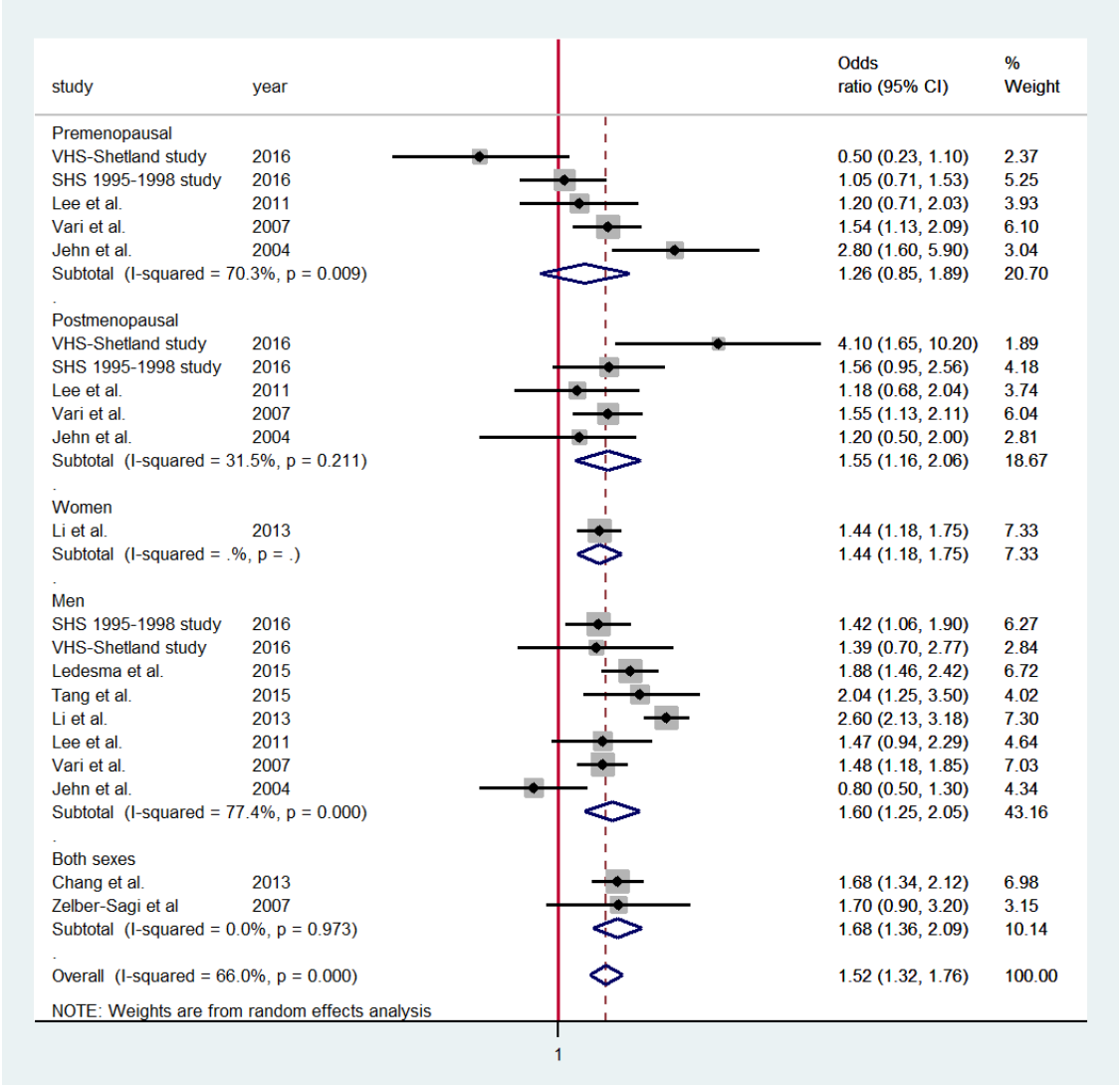
Studies are stratified by sex, menopausal status or both sexes according to the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models. *VHSS-Shetland study = VIKING study.*

Figure 2. Forest plot describing the association between ferritin and high fasting glucose in observational studies.



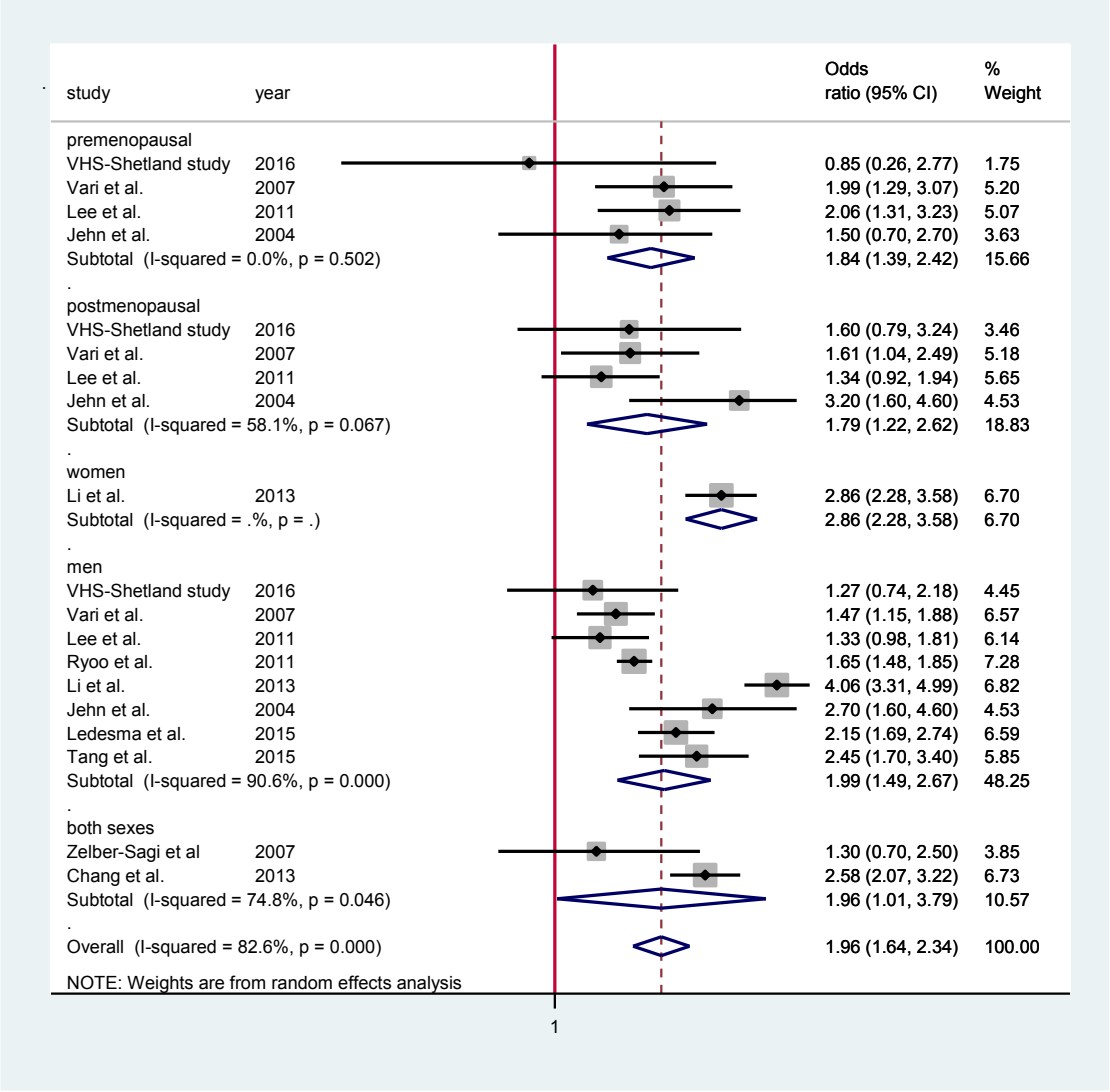
Studies are stratified by sex, menopausal status or both sexes according to the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models. *VHSS-Shetland study = VIKING study.*

Figure 3. Forest plot describing the association between ferritin and increased waist circumference in observational studies.



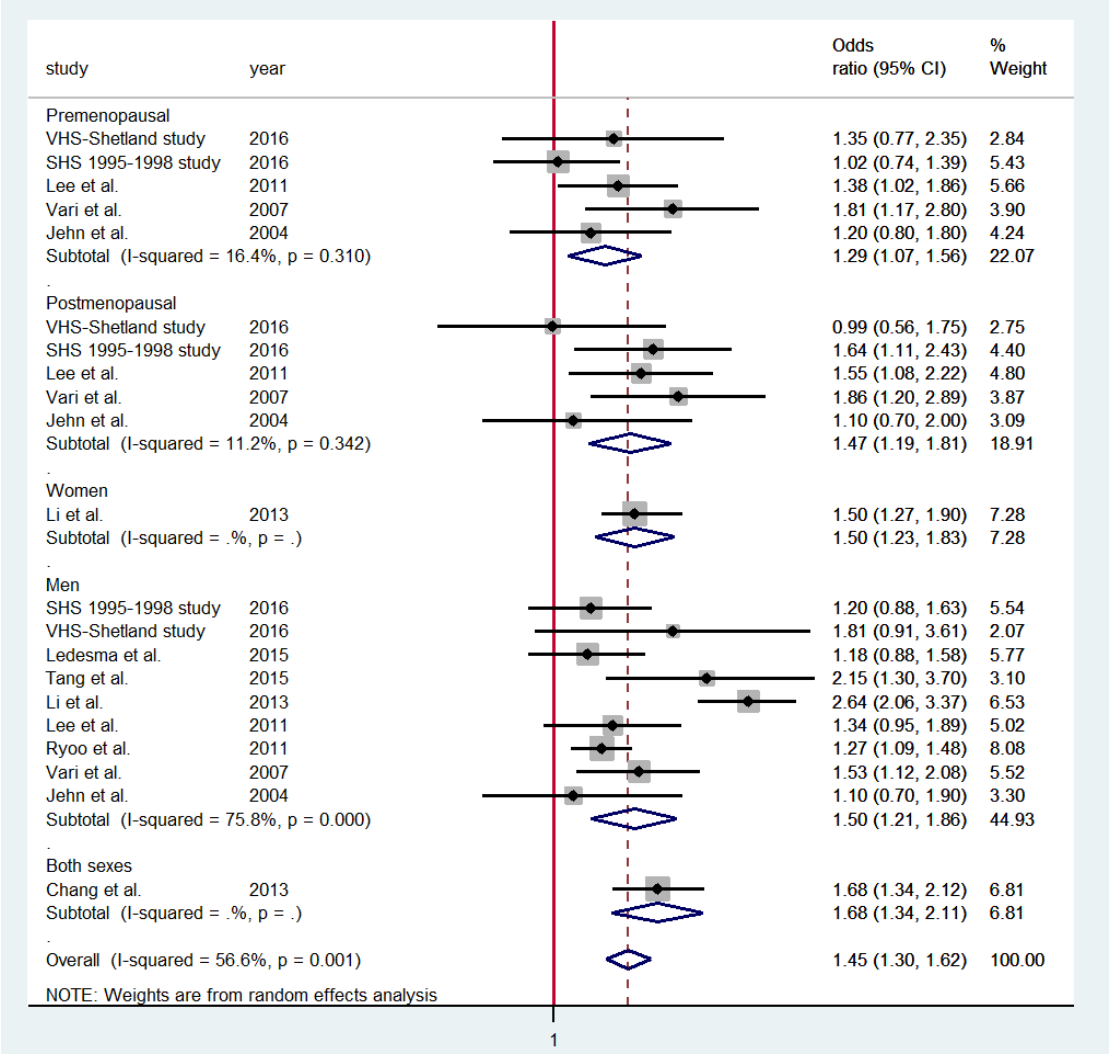
Studies are stratified by sex, menopausal status or both sexes according to the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models. *VHSS-Shetland study = VIKING study.*

Figure 4. Forest plot describing the association between ferritin and high triglycerides in observational studies.



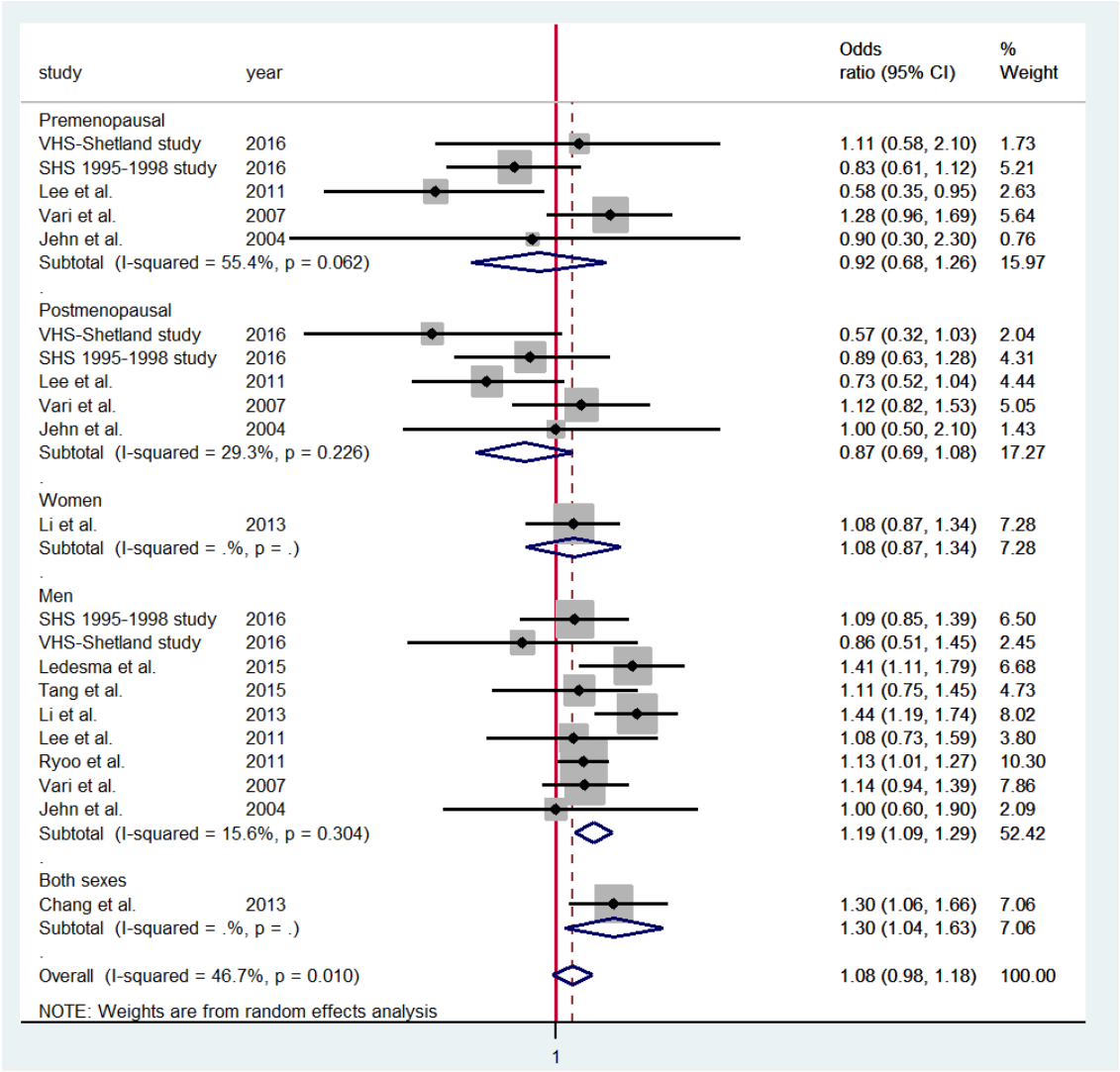
Studies are stratified by sex, menopausal status or both sexes according to the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models. *VHSS-Shetland study = VIKING study.*

Figure 5. Forest plot describing the association between ferritin and low HDL cholesterol in observational studies.



Studies are stratified by sex, menopausal status or both sexes according to the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models.

Figure 6. Forest plot describing the association between ferritin and high blood pressure in observational studies.



Studies are stratified by sex, menopausal status or both sexes according to the way the association was reported in each article. Diamonds are pooled estimates from inverse variance weighted effects random models.

Figure 7. Identification of the cohort SHeS 95-98 (analysis Chapter 5)

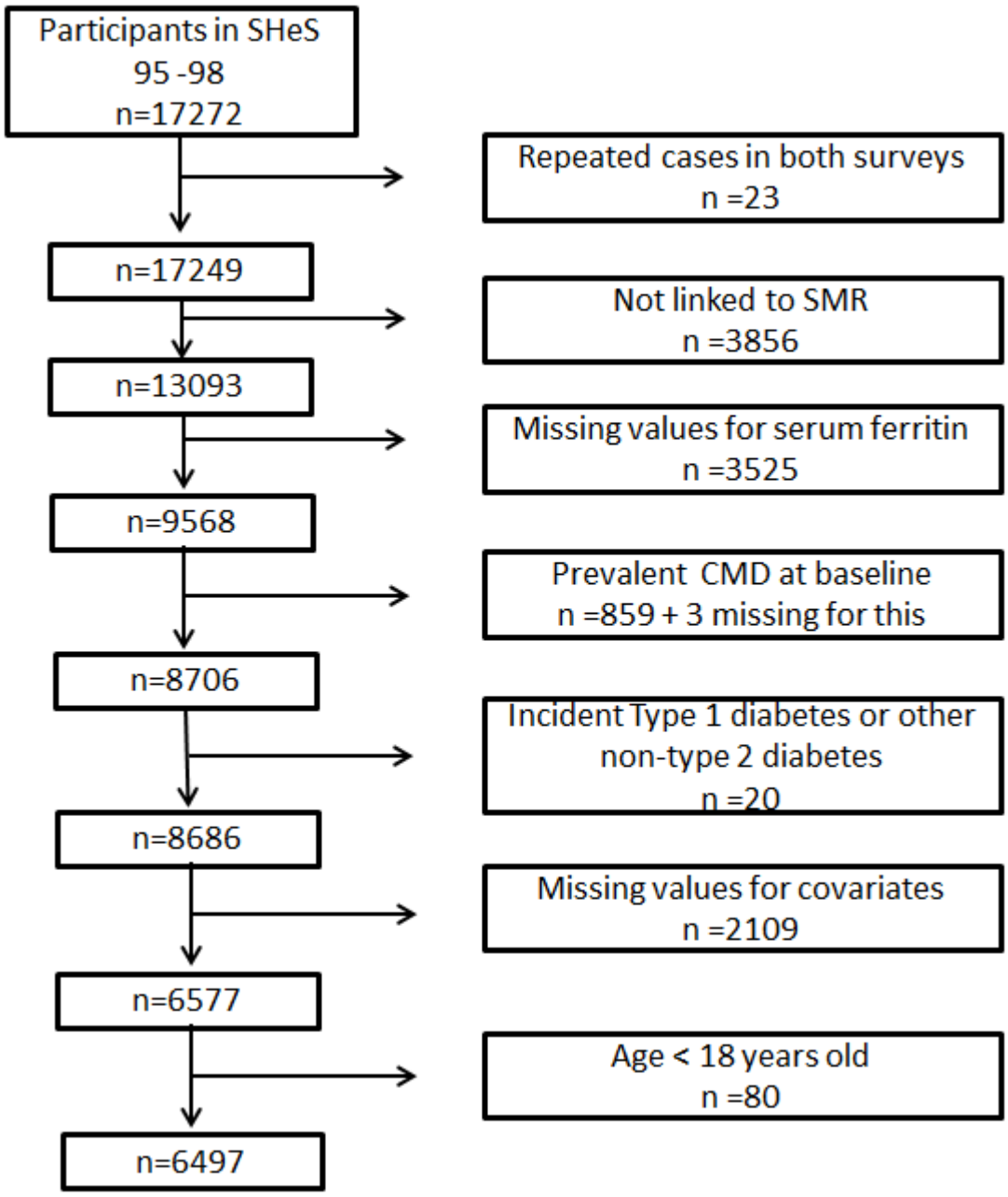
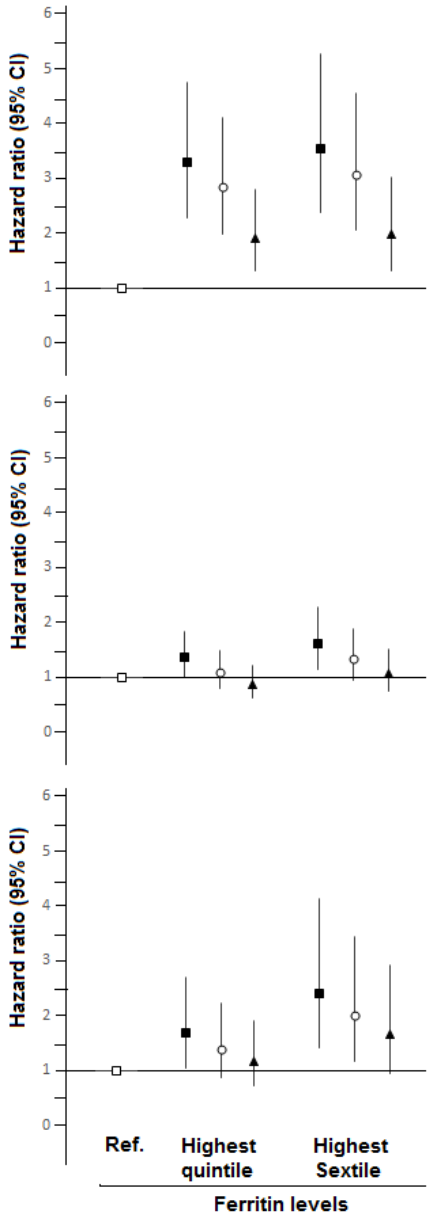


Figure 8. Risk of type 2 diabetes (T2D), coronary heart disease (CHD) and cerebrovascular disease (CEVD) by several sex-specific upper (v. lowest) quantiles of ferritin levels



Ferritin highest quintile: Premenopausal women (Pre-MW) 53-950 µg/L, Postmenopausal women (Post-M) 102-1000 µg/L, Men 169-2251 µg/L. Ferritin highest sextile: Pre-MW 58-950 µg/L, Post-MW 114-1000 µg/L, Men 183-2251 µg/L. □ Reference (lowest quintile, sextile). ■ Unadjusted. ○ Adjusted for age and sex/menopausal status. ▲ Adjusted for age, sex/menopausal status, fibrinogen levels, GGT levels, alcohol intake, smoking, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, body mass index and year of survey. The above analysis included survey weights.

Appendix Newcastle - Ottawa Quality Assessment Scale

(Version modified by Orban and Huth (2014) and adapted for this systematic review/ meta-analysis)

CASE CONTROL and CROSS-SECTIONAL STUDIES

Note: A study can be awarded a maximum of one star (#) for each numbered item within the categories. The maximum score that can be reached is 7 stars.

Selection

1) Representativeness of the cases

a) consecutive or obviously representative series of cases #
b) potential for selection bias
c) no description

2) Selection of controls

a) controls from the same source population as cases #
b) controls from different source population than cases
c) no description

3) Definition of controls

a) general population #
b) having any particular condition or disease
c) no description

Exposure

1) Ascertainment of exposure

a) Method for ferritin measurement described and the according intra-assay or inter-assay coefficient of variation (CV)<15% #
b) Method of ferritin measurement not described or intra-assay or inter-assay CV reported ≥15%
c) No description

2) Same method of ascertainment for cases and controls (especially same time point and same laboratory)

a) yes (assumed if cases and controls are from the same cross-sectional study) #
b) no
c) no description

Outcome

1) Is the case definition adequate?

a) Definition used of metabolic syndrome described and methods of blood sample processing and biochemical and clinical measurements described with at least two readings of blood pressure, and at least two kind of medications recorded among anti-hypertensive, hypoglycaemic, and lipid-lowering agents to complement components of high blood pressure, glucose and triglycerides**#.**
b) based on self report only
c) no description

Comparability of cases and controls

1) Consideration of the most important confounding factors in the design or analysis by matching or adjustment

a) study controls additionally for at least one inflammatory marker and body mass index #

b) study did not adjust at least for the above factors

COHORT STUDIES

Note: A study can be awarded a maximum of one star (#) for each numbered item within the categories. The maximum score that can be reached is 7 stars.

Selection

1) Representativeness of the cohort

a) representative of the general population #
b) selected group, e.g. nurses, volunteers
c) no description of the derivation of the cohort

2) Demonstration that outcome of interest was not present at start of study

a) yes #
b) no

Exposure

1) Ascertainment of exposure

a) Method for ferritin measurement described and the according intra-assay or inter-assay coefficient of variation (CV)<15% #
b) Method of ferritin measurement not described or intra-assay or inter-assay CV reported and ≥15%
c) No description

Outcome

1) Assessment of outcome

a) Definition of metabolic syndrome described and methods of blood sample processing and biochemical and clinical measurements described, with at least two readings of blood pressure, and at least two kind of medications recorded among anti-hypertensive, hypoglycaemic, and lipid-lowering agents to complement components of high blood pressure, glucose and triglycerides**#.**
b) based on self report only
c) no description

2) Was follow-up long enough for outcomes to occur?

a) yes (follow-up was at least three years) #
b) no
c) no description

3) Adequacy of follow-up of cohorts

a) complete follow-up – all subjects accounted for #
b) subjects lost to follow-up unlikely to introduce bias: ≥ 80% follow-up, or complete description provided of those lost #
c) follow-up rate < 80% and no description of those lost
d) no description

Comparability of exposed and unexposed participants

1) Consideration of the most important confounding factors in the design or analysis

a) study controls additionally for at least one inflammatory marker and body mass index #
b) study did not adjust at least for the above factors

Appendix Abstract Poster presentation published in Journal of Epidemiology and Community Health

challenges, evidence that PNs can deliver an integrated approach indicates feasibility of the ENHANCE consultation within routine primary care. Facilitators to that integration will be discussed.

P03 SPIROMETRY AND SURVIVAL IN LARGE UK POPULATION SAMPLES OF LIFELONG NON-SMOKERS

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10.1136/jech-2016-208064.102

Background Reduced ventilatory function is an established predictor of all-cause mortality in the general population. We sought to verify this among lifelong non-smokers and investigate the association with circulatory disease and cancer deaths using mortality follow-up from UK national population surveys.
Methods In UK Biobank, among 149,348 white never-smokers aged 40–69 years at entry, there were 1,357 deaths over a mean 4.6 years of follow-up. In the Health Surveys for England (HSE) 1995, 1996, 2001 and Scottish Health Surveys (SHeS) 1998 and 2003 combined there were 503 deaths among 6,604 white never-smokers aged 40–69 at entry, followed for a mean 14.3 years. Spirometry reference equations (Global Lung Initiative 2012) were used to derive age-sex-height- adjusted standard deviation (z) scores for forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC). The associations of z-scores for FEV1 and FVC with deaths from all causes, circulatory disease and cancers were examined in Stata using proportional hazards models adjusted for age, sex, standing height, socio-economic status, region and survey.
Results In the HSE and SHeS combined dataset, decreasing z-scores for FEV1 and FVC were each associated to a similar degree with increased all-cause mortality (hazard ratios per SD decrement 1.17, 95% CI 1.09–1.25 for zFEV1 and 1.19, 1.10–1.27 for zFVC). This was replicated in UK Biobank (HRs per SD 1.24, 1.18–1.30 and 1.27, 1.20–1.33, respectively). In HSE-SHeS, zFEV1 and zFVC were also associated to similar degrees with mortality from circulatory diseases (HRs per SD 1.21, 1.05–1.38 for zFEV1 and 1.22, 1.06–1.40 for zFVC). These associations were stronger in Biobank (1.48, 1.33–1.65 and 1.52, 1.36–1.70, respectively). For cancer mortality, corresponding hazard ratios were more consistent: 1.10, 1.00–1.22 for zFEV1 and 1.12, 1.01–1.24 for zFVC in HSE-SHeS and 1.11, 1.05–1.18 and 1.13, 1.06–1.20, respectively, in Biobank. Among 102,950 white never-smokers in Biobank with spirograms most closely approximating internationally recommended acceptability and reproducibility criteria, of whom 881 died, z-scores for the FEV1/FVC ratio were significantly associated with all-cause mortality when modelled jointly with zFEV1 but z-scores for FEV1/FVC were not associated with survival independent of zFVC.
Conclusion FEV1 and FVC each predict all-cause mortality, circulatory disease mortality and cancer mortality among white lifelong non-smokers in the UK. In those who are able to complete spirometry to stringent international standards, FVC is a more influential predictor than FEV1. However, in settings where end-blow quality is difficult to assess, FEV1 is the more generally applicable predictor.

P04 MEDIATORS OF THE ASSOCIATION BETWEEN SOCIO-ECONOMIC POSITION AND TYPE TWO DIABETES USING THE BRITISH HOUSEHOLD PANEL SURVEY

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10.1136/jech-2016-208064.103

Background Socio-economic inequalities exist in prevalence and incidence of type-two diabetes (T2D). Hypothesised explanations in the literature refer to differences on the basis of socio-economic position (SEP) in experience of material deprivation, psychosocial stress and health behaviours. We test the hypothesis that each of these pathways mediated the association between SEP and T2D, and vary on the basis of area.
Methods Using the British Household Panel Survey (BHPS), a longitudinal study which begun in 1991, we looked at factors influencing self-reported T2D incidence. The sample was restricted to participants satisfying the following criteria: a) British residents of white ethnicity; b) non-diabetic; c) aged 30–70 on entry to the survey; and d) present in waves 10–18 of the survey. These criteria result in an analytical sample of 6,641 participants. The association between T2D and SEP is decomposed into direct and indirect effects using the Karlson, Holm and Breen (KHB) decomposition technique. Models with random effects for area were run separately for England, Scotland and Wales – due to differences in deprivation scores – using Stata 14.
Results Evidence was found for an association between SEP and T2D. The total effect (coefficient (β) 0.320, standard error (SE) 0.171) was decomposed into direct and indirect effects through the material, psychosocial and health behaviour pathways. The contribution of these mediators to the total effect was significant and explained approximately one third of the total effect. This was mostly explained by health behaviours; exercise (β 0.05, SE 0.02) and obesity (β 0.04, SE 0.02). The material and psychosocial pathways did not contribute significantly. Random effects for area did not improve the model fit for each region ($p > 0.05$).
Conclusion These findings show the association between SEP and T2D is mediated by exercise and obesity, confirming the findings of previous studies. This study was limited by imperfect measures of SEP and each pathway. Policy interventions aimed at promoting healthy lifestyle could focus on making this accessible to low SEP groups in order to reduce inequalities in T2D. Further research in this area would benefit from measuring a greater array of health behaviour.

P05 BODY IRON STORES AND METABOLIC SYNDROME: CROSS SECTIONAL STUDY IN A UK POPULATION

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10.1136/jech-2016-208064.104

Background An association has been described between elevated ferritin (the major iron storage protein) levels and metabolic syndrome (MetS) in several populations. However there are limited data on this association in the UK population. We investigated whether ferritin levels were associated with MetS and its components in participants in the Viking Health Study – Shetland (VHSS).
Methods The analysis was conducted using data collected between 2013 and 2015 for 2047 individuals from the Shetland Islands (589 premenopausal women, 625 postmenopausal

women, and 833 men) of 18–93 years of age. Ferritin levels were categorised into sex/menopausal specific quartiles and cut- points from the consensus definition of MetS and the interim joint statement definition of MetS was used to define risk factors. Logistic regression models were used to adjust for age, fibrinogen levels, smoking, alcohol consumption and body mass index.

Results Median (inter-quartile ranges) were 51 (38.9–63.2) years for age, and 56 (29–97) µg/L for ferritin. Prevalence of risk factors were: high waist circumference (HWC) 66.7 %, low HDL-cholesterol (LHDL) 17.1%, high glucose (HG) 7.2%, high triglycerides (HT) 11.9% and high blood pressure (HBP), 50.4% and prevalence of MetS (having three or more of the previous risk factors) was 18%. In un-adjusted models, the highest compared with the lowest quartile of ferritin was associated with HT and HWC in post-menopausal women ($P < 0.05$) and with all of the risk factors (except HBP) in men ($P < 0.05$). In men the crude OR for MetS for the highest compared to the lowest quartile of ferritin was 2.15 (1.36–3.41) ($P < 0.001$) but the fully adjusted OR was 1.43 (0.83–2.46) ($P = 0.207$). The association between ferritin and MetS was not statistically significant in either group of women in crude or adjusted analyses.

Conclusion Ferritin levels were not independently associated with MetS in this population. The analysis confirms our previous findings of the lack of an independent association between increased ferritin and some MetS components (LHDL, HBP and HWC) among participants in Scottish Health Surveys 1995–1998. The contrasting findings with previous studies, suggest that the ferritin-MetS association could differ between populations, possibly related to differences in distribution of ferritin and a threshold effect.

P06 CARDIOVASCULAR RISK CALCULATORS ARE INCONSISTENT IN INCLUDING ETHNICITY AS A PREDICTOR

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10.1136/jech-2016-208064.105

Background There are several calculators available internationally for predicting risk of cardio-vascular disease based on an individual's particular risk factors. Ethnicity is associated with risk of cardio-vascular disease. We studied 10 important calculators and considered (a) whether ethnicity was included as a risk factor (b) which ethnic groups were included within each calculator; and (c) the mathematics by which ethnicity was incorporated.

Methods Ten commonly used calculators were identified by examining the literature and searching risk prediction websites and examined in detail through both the published literature and online technical descriptions. Their component risk factors were tabulated and a comparison made.

Results Of the ten calculators examined, seven did not include ethnicity as a risk factor. Those seven calculators that excluded ethnicity, included risk factors which have the potential to account for some of variation in risk associated with ethnicity

In the three calculators that included ethnicity, there is variation as to which ethnic groups are considered, and this probably reflects geographic variation in the important ethnic groups for that country or region.

In the three calculators that included ethnicity as an additional risk factor, there was inconsistency in the mechanism by which ethnicity was incorporated. In one it was a fixed additive term, in another it was a full interaction with other risk factors presented as a distinct model. In the third an additive term but with an upper bound of risk meaning implying ethnicity interacts with other risk factors.

Conclusion Ethnicity is often not always included and when it is it is included it is done so in different ways in different calculators. This diversity suggests that there remains uncertainty as to what and how risk factors should be included and that ethnicity itself isn't agreed as an essential additional variable. Refinement of calculators will likely arise as the acquisition of prospective multi-ethnic data sets improves.

P07 MEDIA CONSTRUCTIONS OF 'ARTHRITIS': A MIXED METHODS QUALITATIVE STUDY

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10.1136/jech-2016-208064.106

Background Musculoskeletal conditions, including arthritis, have a global impact, causing increased disability and reduced quality of life. Previous research has demonstrated negative attitudes and beliefs about arthritis exist which means the condition is often undermanaged and deprioritised. One potential influence on such attitudes is the media. Understanding how the media constructs arthritis, and what impact media constructions have on perceptions of arthritis, will shed light on factors that influence attitudes and management of the condition in everyday life.

Methods This research aimed to investigate media constructions of arthritis. Mixed Methods were used, including media analysis of highest circulating newspapers ($n = 11$) and magazines ($n = 3$), and focus groups ($n = 2$) to explore reception of media messages. Results were analysed using a combination of thematic, discourse and imagery analyses.

Results A total of 1014 newspaper and 18 magazine articles were analysed. Arthritis was conceptualised in three ways – as a disease, condition or ailment. As such, arthritis was not presented as a singular condition; instead the construction, enactment and reality of arthritis were multiple. These multiple conceptualisations were shaped by wider social issues, such as understandings of disability (saints or scroungers) and ageing (peril or promise), and their representation in the media was determined by factors of media production (audience targeting, commercial interests and 'newsworthiness'). The focus group findings reflected these perceptions, as well as illustrating that media trust and credibility influence how media messages are received and interpreted by the general public.

Conclusion Recognising arthritis as multiple is important for health care professionals and patients, as the multiple conceptualisations can impact on how arthritis is enacted, and may affect perceptions of legitimacy and deservedness. Media representations of arthritis may lead to the condition being