UNIVERSIDAD D CÓRDOBA

Ceruloplasmina y su implicación en la enfermedad cardiovascular

Ceruloplasmin and its involvement in cardiovascular disease

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TÍTULO DE LA TESIS: CERULOPLASMINA Y SU IMPLICACIÓN EN LA ENFERMEDAD CARDIOVASCULAR.

DOCTORANDO/A: ANTONIO PABLO ARENAS DE LARRIVA

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

D. JAVIER DELGADO LISTA, PROFESOR TITULAR DE LA UNIVERSIDAD DE CÓRDOBA; ÁLVARO ALONSO GUTIÉRREZ, CATEDRÁTICO DE LA UNIVERSIDAD DE EMORY, ATLANTA, Y D. JOSÉ LÓPEZ MIRANDA, CATEDRÁTICO DE MEDICINA DE LA UNIVERSIDAD DE CÓRDOBA,

HACEN CONSTAR:

Que el trabajo titulado "CERULOPLASMINA Y SU IMPLICACIÓN EN LA ENFERMEDAD CARDIOVASCULAR" ha sido realizado por D. Antonio Pablo Arenas de Larriva, bajo nuestra dirección, en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), dentro del grupo GC9: Nutrigenómica. Síndrome Metabólico. Este trabajo ha conseguido un nivel científico de suficiente relevancia como para derivar en la publicación de tres artículos en revistas internacionales en Q1 de sus categorías:

- ***Cardiology and Cardiovascular Medicine.*** Int J Cardiol. 2017;241:223-8. DOI: 10.1016/j.ijcard.2017.04.005 Journal Impact Factor (2017): 4.034. (Q1),
- ***Hematology.*** Journal of Thrombosis and Haemostasis. 2019;17(5):818-26. DOI: 10.1111/jth.14420. Journal Impact Factor (2019): 4.157. (Q1),
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A nuestro juicio reúne los méritos suficientes para ser defendido ante el tribunal correspondiente y poder optar al grado de Doctor.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 29 de Noviembre de 2021

Firma de los directores

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ABSTRACT

INTRODUCTION

Inflammation and oxidative stress play a key role in the initiation and maintenance of atherosclerosis and are related to the occurrence of events leading to vessel occlusion, both in the venous system (thromboembolic disease) and in the arterial system (cardiovascular disease). Although there are no clear theories on how these occlusive diseases in both arterial and venous territories are linked, they share the increase of certain inflammatory and oxidative-stress markers. Ceruloplasmin (CP) is a protein involved in copper metabolism that has also been linked to inflammatory responses, whereas the association to occlusive vascular diseases has not been fully characterized.

HYPOTHESIS

Our hypothesis is that high levels of CP would be associated with the incidence of cardiovascular events.

OBJECTIVES

1) To evaluate whether high levels of plasma CP are associated with increased incidence of atrial fibrillation.

2) To evaluate whether high levels of plasma CP are associated with increased incidence of venous thromboembolism.

3) To perform a systematic review of the current evidence on whether high levels of plasma CP are associated to a higher risk of coronary heart disease.

METHODS

a) We conducted a first analysis evaluating the association of CP with AF incidence in a large cohort, the Atherosclerosis Risk in Communities (ARIC) Study.

b) We conducted other investigation to test the association between CP and VTE incidence in the same ARIC study population.

c) Finally, to evaluate the influence of CP on CHD, we conducted a systematic review exploring the impact of CP on the risk of CHD over the last three decades.

RESULTS

Higher plasma CP levels were associated with incident AF in the ARIC cohort. Regarding VTE, higher concentrations of plasma CP were also associated with greater incident VTE

rates. Finally, most of 18 eligible studies reviewed supported a direct relationship between CP elevated levels and incidence of CHD.

CONCLUSIONS

We have evaluated the association between CP and three highly prevalent diseases derived from alterations in the cardiovascular system, both venous and arterial location, establishing that high CP levels are related to the occurrence of these conditions.

INTRODUCCIÓN

La inflamación y el estrés oxidativo desempeñan un papel fundamental en el inicio y desarrollo del proceso aterosclerótico y están relacionados con la aparición de eventos que conducen a la oclusión de los vasos, tanto en el sistema venoso (enfermedad tromboembólica) como en el arterial (enfermedad cardiovascular). Aunque no hay teorías claras sobre cómo se relacionan estas enfermedades oclusivas en los territorios arteriales y venosos, comparten la elevación de ciertos marcadores inflamatorios y de estrés oxidativo. La ceruloplasmina (CP) es una proteína que actúa en el metabolismo del cobre que se ha relacionado con la respuesta inflamatoria, la cual no se relacionado hasta el momento con las enfermedades vasculares oclusivas.

HIPÓTESIS

Nuestra hipótesis es que altos niveles de CP estarían implicados en la aparición de nuevos eventos cardiovasculares.

OBJETIVOS

1) Evaluar si niveles elevados de CP en plasma se asocian a una mayor incidencia de fibrilación auricular.

2) Evaluar si niveles elevados de CP en plasma se asocian a una mayor incidencia de tromboembolismo venoso.

3) Realizar una revisión sistemática de la evidencia actual sobre si niveles elevados de CP en plasma se asocian a un mayor riesgo de enfermedad coronaria.

MÉTODOS

a) En un primer análisis, evaluamos la asociación de la CP con la incidencia de FA en el estudio ARIC (Atherosclerosis Risk in Communities), una cohorte comunitaria.

b) Realizamos otro trabajo de investigación para comprobar la asociación de CP e incidencia de ETV en la misma población.

c) Por último, para evaluar la influencia de la CP en la cardiopatía isquémica, realizamos una revisión sistemática en la que exploramos el impacto de la CP en el riesgo de cardiopatía isquémica en las tres últimas décadas.

RESULTADOS

Niveles más altos de CP circulante se asociaron con mayor incidencia de FA en la cohorte ARIC. En relación con la ETV, las mayores concentraciones de CP circulante también se asociaron con mayores tasas de ETV. Por último, la mayoría de los 18 estudios revisados

apoyaron una relación directa entre niveles elevados de CP y la incidencia de cardiopatía isquémica.

CONCLUSIONES

Tras evaluar la asociación entre la CP y tres enfermedades altamente prevalentes derivadas de alteraciones del sistema cardiovascular, tanto de localización venosa como arterial, podemos concluir que niveles elevados de CP están relacionados con la aparición de estas enfermedades.

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Chapter 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

Cardiovascular disease remains the leading cause of mortality worldwide highlighting the need of the development of new approaches to better understand the underlying pathophysiology. Arterial and venous thrombotic conditions are two wide groups inside the spectrum of cardiovascular diseases. Ischemic heart disease and ischemic stroke comprise the major arterial thromboses, and deep-vein thrombosis and pulmonary embolism comprise venous thromboembolism. Atrial fibrillation is a major risk factor for stroke and systemic arterial thromboembolism and its link to VTE is now being investigated (1).

CHD remains a substantial public health challenge worldwide. An estimated 126.5 million individuals lived with CHD and 10.6 million new CHD cases occurred in 2017, contributing to 8.9 million deaths (2).

Many individuals in the general population have one or more risk factors for CHD. The top three potential modifiable risk factors for CHD deaths in 2017 were dietary risks, high systolic blood pressure and high LDL cholesterol and along with diabetes and smoking, are estimated to be responsible for more than half of cardiovascular mortality (3). Although current guideline-guided CHD therapy has lowered both recurrence and death rates, people with CHD remain at high risk for these complications. One third of all CHD with known, controlled risk factors will have a recurrence in the following 10 years (4). This is called residual risk, and many approaches have been taken to tackle it.

In relation to the venous system, VTE is the third most common cardiovascular disease after CHD and stroke (5). VTE can manifest as an isolated lower extremity deep vein thrombosis (DVT) or a clot can break off from the lower extremities and travel to the lung to present as a pulmonary embolism (PE).

The incidence of PE in the general population is estimated at 39-115/100,000 person-years, and of DVT at 53-162/100,000 person-years. It is eight times higher in people in their eighties than in those in their fifties. Worldwide, about 10 million cases of VTE are estimated to occur annually. In the United States, about 676,000 DVTs, 340,000 PEs and 1,016,000 total VTE events and about 60,000-100,000 deaths annually (for a population of 319 million) are estimated (1, 5-7). According to the most recent mortality data in Europe (WHO data), the average yearly number of PE-related fatalities

in Europe is 38,929 (for a population of 651 million in 2015). At the same time, a progressive decrease in European mortality due to PE is observed (from 12.8% in 2000 to 6.5% in 2015; annual mortality rate decrease of 5.0%). These same figures referring to Spain provide 2,232 annual deaths (2015) (8). In the period 2004-2017, in-hospital mortality due to PE in the National Health System decreased from 11.6% to 6.2%14. Such declining mortality is also confirmed by the Registro Informático de pacientes con Enfermedad Tromboembólica (RIETE) (30-day mortality in PE decreased from 6.6% (2001-2005) to 4.9% (2010-2013)). The latest data from this registry (March 1, 2020; 44,482 patients with PE) show an (unadjusted) mortality of 5.2%. This admits important nuances according to age, as it is 3 times higher in patients > 81 years (9.2%) compared to those aged 36-60 years (3.1%). For DVT (36,540 patients), 30-day mortality is 2.6% (9).

AF is the most frequent heart rhythm disbalance. It has been estimated that 6–12 million people will suffer this condition in the US by 2050 and 17.9 million people in Europe by 2060 (10, 11). AF shares strong epidemiological associations with other conditions such as heart valve disease, diabetes mellitus, arterial hypertension, and overweight/obesity, as well as with metabolic syndrome and its components sleep apnea and inflammation (12-14). Genetic factors can also be involved, and more recent data have focused on modifiable lifestyle factors such as alcohol consumption and physical exercise (15). As we commented, AF is a major risk factor for ischemic stroke and systemic arterial thromboembolism producing important economic burden along with significant morbidity and mortality. The prevalence of AF is very low among young individuals (<1% in people aged <40 years) but increases with age, reaching between 10% and 17% in those aged >80 years. With the worldwide aging of the population, an AF epidemic is expected within the next years (16).

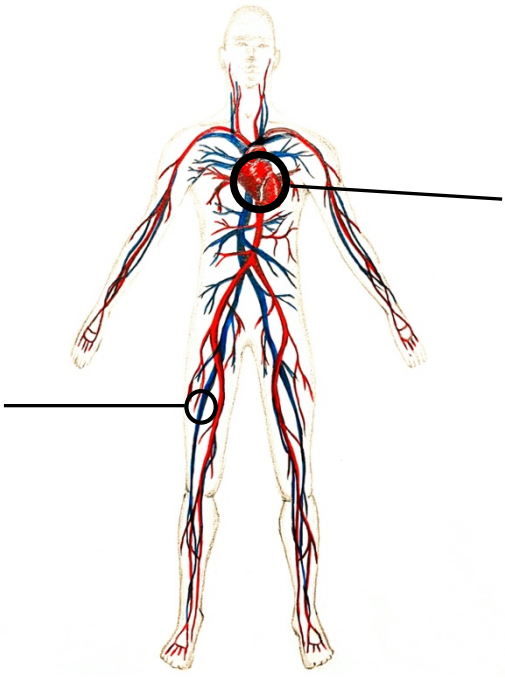
These three diseases previously mentioned (CHD, VTE and AF), have a huge clinical, epidemiological and economic relevance, and it is necessary the search for additional biomarkers which may help to detect or be an early predictor of those cases which will develop a worse prognosis despite controlled risk factors.

In line with the above, we aimed to investigate if CP is a biomarker associated to the development of these conditions.

CP is an enzyme synthesized in the liver that is responsible for transport of circulating copper and is also involved in iron metabolism. It is an acute-phase reactant that may have antioxidant actions, but it can also participate in the generation of free radicals, leading to an inflammatory state, which has been linked to the pathophysiology of all vascular diseases (17, 18).

Oxidative stress might play a central role in the initiation of CHD, but it remains unclear whether proteins involved in this process, like CP, act as passive markers of inflammation or they are causal mediators in its development. In the same line, several studies have demonstrated a relationship between different proteins involved in inflammatory processes and VTE or AF. Inflammation seems to trigger a chain reaction whereby procoagulant factors are activated and the fibrinolytic pathway is inhibited. For example, the Atherosclerosis Risk in Communities (ARIC) study previously found that elevated high-sensitivity C-reactive protein, but not fibrinogen, was independently associated with an increased risk of VTE (19). Likewise, in AF, CP appears to promote structural changes in the atrium making it more arrhythmogenic (20). If this relationship between AF or VTE and CP is confirmed, new prevention approaches could be researched, and we could identify individuals at increased risk of AF or VTE.

To summarize, the purpose of this thesis is to deepen our understanding of the relationship between the main cardiovascular diseases (CHD, VTE, AF) and CP. CP is a biomarker that is readily available in clinical practice, and, if this relationship is shown, it would open new lines of research to unveil if there is a causal relationship behind the association, or CP is just a marker increased in those diseases.



Coronary heart disease and ischemic stroke comprise the major arterial thrombosis

Atrial fibrillation is a major risk factor for stroke and systemic arterial thromboembolism

Deep venous thrombosis and pulmonary embolism comprise **venous thromboembolism**

Chapter 2

REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

A. Structure and biological role of ceruloplasmin

CP is a 2-glycoprotein fraction of plasma proteins identified by Holmberg and Laurell in 1948. Although it is produced in the liver, extrahepatic expression has been found in the brain, lungs, spleen, and testicles.

CP has a molecular weight of around 132 kDa. The CP gene is located in the 3q25 region of chromosome 3. This protein is part of the blue multinuclear copper oxidases family. It comprises a single polypeptide chain of 1046 amino acids. Six plastocyanin domains are organized in a triangle array in the molecule. Six copper atoms are present, three of which form a trinuclear cluster at the domain 1–6 interface and the other three of which form mononuclear sites in domains 2, 4, and 6. The trinuclear center and the mononuclear copper in domain 6 form a cluster that resembles that of ascorbate oxidase (Figure 2) (21).

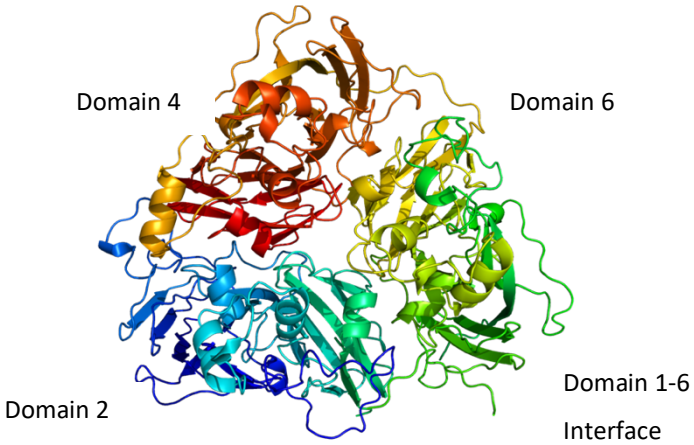


Figure 2. Ceruloplasmin structure. Adapted from C.W. Linder (22)

The main function of CP is transport and distribution of copper to tissues. Copper is needed by cells for a variety of proteins with defensive functions, including cytochrome c, metallothioneins, and other oxidases (23). About 95% of total circulating copper is associated to CP and this protein has been found in a variety of tissues and

cells, including erythrocytes, aorta and heart, liver endothelium, leukocytes, Kupffer cells, and human placental cells. The membrane galactosyl recognition mechanism links CP to endothelial cells (24-26).

Copper transport from blood to tissues is tightly regulated by copper chaperone proteins at the plasma membrane and in the intracellular compartment due to its highly reactive nature. As a result, in non-pathological situations, there is almost no free (unbound) copper in the cytoplasm of cells (27).

CP has ferroxidase activity, which means it can convert dangerous ferrous ions to less harmful ferric ions. The Fenton reaction, which employs Fe^{2+} to generate ROS, is assumed to be inhibited by the oxidation of Fe^{2+} to Fe^{3+} by CP, which is expected to minimize oxidative stress. The release of iron from cellular reserves for uptake by the circulatory iron transport protein transferrin is likewise dependent on this event. Iron accumulates in the brain, liver, and pancreas in patients who do not have CP or in CP knock-out mice, which can lead to diabetes and dementia (28-30).

Apart from playing a role in copper and iron metabolism, CP is an acute-phase reactant that may work as an antioxidant but can also generate free radicals. A modulating action has been described in processes such as coagulation, angiogenesis, as well as an inactivating capacity of biogenic amines and defense against oxidative stress (31-33). It is also part of the family of inflammation-sensitive proteins that includes α_1-antitrypsin, haptoglobin, orosomucoid and fibrinogen (34) whose levels have been associated with cardiovascular risk factors such as hypercholesterolemia, body weight gain, diabetes and high blood pressure (35).

Normal values for serum CP are different by age. They are very low during early infancy, then peak in early childhood (approximately 300 to 500 mg/L), and then decline to the adult range (200 to 350 mg/L). CP is estrogen-sensitive, and levels are elevated in pregnancy and in patients on hormonal supplementation.

B. Inflammation markers, ceruloplasmin and coronary heart disease

Inflammation is central to understanding the pathogenesis of atherosclerosis, and is implicated both in the development and in the rupture of the atherosclerotic plaque that causes cardiovascular events (36-39).

Many inflammatory markers to date have been associated to atherosclerosis. CRP, IL-6 or leukocyte enzyme myeloperoxidase are among the most widely studied. CRP is probably the most extensively studied. It has been suggested that there could be a direct effect of CRP on the development of atherosclerosis, based on the finding of CRP in atherosclerotic lesions (40-49). IL-6 signals a downstream proinflammatory response by activating membrane-bound IL-6 receptors (IL-6R) on the cell surface. IL-6 and IL-6R appear to have a direct causal role in the development of CHD and may be a future target for therapeutic interventions to prevent CHD (50). Finally, leukocyte enzyme myeloperoxidase is another inflammatory marker which has been associated with the presence of coronary disease and may be predictive of the presence of acute coronary syndrome in patients with chest pain (51-54).

Cardiovascular risk has also been associated with many other markers of inflammation. Elevated levels of white blood cells, erythrocyte sedimentation rates, IL-18, tumor necrosis factor alpha, transforming growth factor beta, soluble intercellular adhesion molecule-1, P-selectin, cathepsin S, and lipoprotein-associated phospholipase A2 have been reported as markers of increased CHD risk (55-60).

Regarding ceruloplasmin, there is not a consensus on its association with cardiovascular disease, or its role in the pathophysiology of atherosclerosis. Thereby, reviewing the available data in a systematic disease was a need that we have covered in the article included in this thesis.

C. Inflammation, ceruloplasmin and venous thrombosis

It has traditionally been thought that venous thrombosis and arterial thrombosis have different etiopathogenesis. While red blood cells and fibrin are the major participants of the “red clot” seen in venous thrombosis, aggregated platelets are the major participants of the “white clot” seen in arterial thrombosis. Venous thrombosis has been associated with hypercoagulability and/or reduced blood flow, whereas arterial thrombosis has been linked to atherosclerosis and platelet activation. However, the classical view of separate mechanisms for arterial and venous thrombosis has been challenged. It has been shown that patients with arterial thrombosis were also at increased risk for venous thrombosis, and overlapping risk factors have been found to

be associated with both arterial and venous thrombotic events (61, 62). Furthermore, it was shown that inflammation and platelet activation are also involved in the pathogenesis of venous thrombosis (63).

The pathogenesis of inflammation-induced thrombosis is complicated. In general, inflammation causes platelet, leukocyte and endothelial cell activation, and the interactions between these cells may result in endothelial injury and endothelial cell dysfunction, causing the loss of physiologic anticoagulant and vasodilatory properties of the normal endothelium. With a simplistic approach, one may accept endothelial injury as the key factor connecting chronic inflammation with thrombosis (64).

Various environmental agents may trigger systemic inflammation in the context of genetic predisposition. Transcription factors and intracellular enzymes including caspase family proteases are activated, followed by the secretion of various inflammatory mediators including cytokines, chemokines and growth factors (figure 3). These inflammatory mediators activate the endothelial cells, leukocytes and platelets, inducing the expression of relevant cell adhesion molecules on their surfaces. Complex interactions between endothelial cells, platelets and leukocytes occur, causing endothelial injury and endothelial cell dysfunction. Non thrombogenic endothelial surface may no longer be maintained in the setting of inflammation. In other words, inflammation transforms the endothelial cells into a prothrombotic and antifibrinolytic phenotype (61, 65-67). However, even if there is no vessel wall and endothelial damage, inflammation itself may trigger venous thrombus formation. Increased tissue factors (TF) expression occurring especially on platelets, endothelial cells and monocytes is an important link connecting inflammation with thrombosis. TF mainly stimulates the formation of thrombin, which has proinflammatory effects. Microparticles (MPs) derived from platelets, endothelial cells and leukocytes are also involved in the development and the amplification of thrombosis. Fusion of MPs with activated platelets results in decryption of TF and the initiation of thrombosis. Platelet MPs are not only prothrombotic, but also inhibit fibrinolysis, delaying thrombus resolution and facilitating thrombus growth (68, 69).

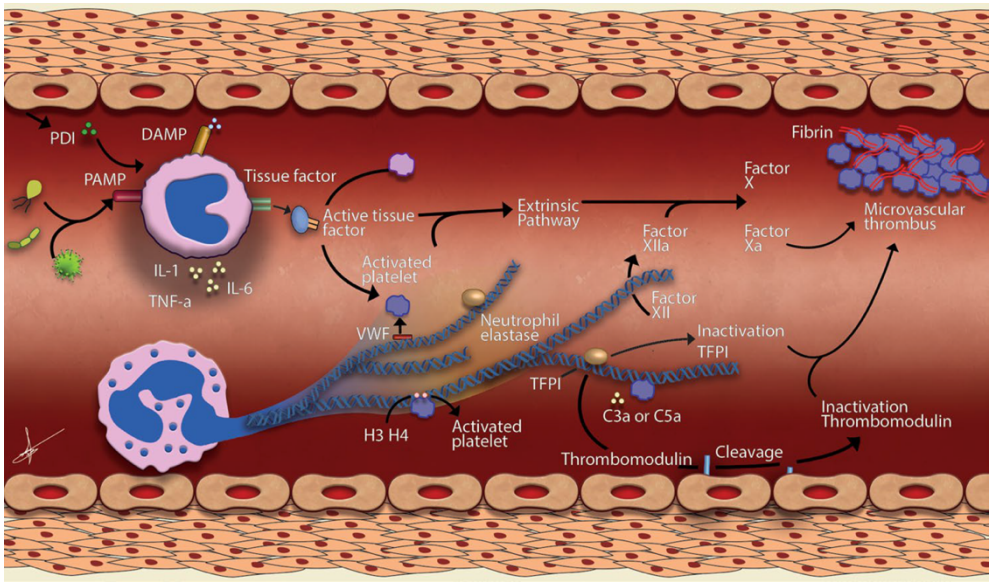


Figure 3. Pathogenic mechanisms connecting inflammation with thrombosis.. Abbreviations: IL-1, interleukin; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; TFPI, tissue factor pathway inhibitor. PAMP: pathogen-associated molecular pattern. DAMP: damage-associated molecular pattern. Adapted from Vázquez-Garza et al., (70)

Several studies have demonstrated a relationship between various proteins involved in inflammatory processes and VTE. For example, the ARIC study previously found that elevated CRP, but not fibrinogen, was independently associated with an increased risk of VTE (19). However, there are others results to the contrary as Sveinsdottir et al. investigation where no significant relationships between VTE incidence and CP or other inflammatory markers (fibrinogen, orosomucoid, α_1-antitrypsin, and haptoglobin) were found (71).

There is a complex interplay between inflammation and the coagulation system and we intend to shed some light on this field exploring whether CP can be involved in VTE.

D. Inflammation, ceruloplasmin and atrial fibrillation

Inflammation may also play a role in the genesis of AF. Measurement of serum CRP has been used to assess the relationship between AF and inflammation.

Observational studies have reported elevated serum levels of CRP in patient populations with any of the following characteristics: later development of AF (72),

history of atrial arrhythmias (73), failed cardioversion (74), recurrence of AF after cardioversion (75), and development of AF after cardiac surgery.

However, inflammation is more likely a marker for other conditions associated with AF, as opposed to being a direct cause or a perpetuating agent. The strongest evidence against a direct causal role for inflammation, as detected by an elevation in CRP, comes from a Mendelian randomization study that evaluated nearly 47,000 individuals in two cohorts from Copenhagen, Demark (76). After multifactorial adjustment, a CRP level in the upper versus lower quintile was associated with a significantly increased risk of the development of AF (HR 1.77, 95% CI 1.22-2.55). Genotype combinations of four CRP single nucleotide polymorphisms (SNPs) were not associated with an increased risk of the development of AF. Thus, inflammation, as determined by CRP, is not likely to be causative of AF.

CP could promote structural changes in the atrium making it more arrhythmogenic. A study showed that higher concentrations of CP in blood were associated with increased AF risk. In this same study, a variant of rs11708215, a single nucleotide polymorphism (SNP) located in the CP gene promoter, was associated with both higher CP concentrations in blood and increased AF risk. Another SNP, rs13072552, also in the CP gene, was associated with CP plasma concentration. (20). These results, however, have not been replicated in larger studies.

Chapter 3

HYPOTHESIS AND OBJECTIVES

CHAPTER 3: HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Our hypothesis is that high levels of plasma ceruloplasmin are associated with the incidence of arterial and venous events.

OBJECTIVES

We studied the influence of plasma ceruloplasmin in the incidence of conditions affecting the vascular system. For this, we set the following objectives:

1) To evaluate whether high levels of plasma ceruloplasmin are associated with increased incidence of atrial fibrillation.

2) To evaluate whether high levels of plasma ceruloplasmin are associated with increased incidence of venous thromboembolism.

3) To perform a systematic review of the current evidence in whether high levels of plasma ceruloplasmin are associated to a higher risk of coronary heart disease.

Chapter 4

PUBLICATIONS FROM THIS TESIS

CHAPTER 4: PUBLICATIONS FROM THIS THESIS

1. **Arenas de Larriva AP**, Norby FL, Chen LY, Soliman EZ, Hoogeveen RC, Arking DE, Loehr LR, Alonso A. CIRCULATING CERULOPLASMIN, CERULOPLASMIN-ASSOCIATED GENES, AND THE INCIDENCE OF ATRIAL FIBRILLATION IN THE ATHEROSCLEROSIS RISK IN COMMUNITIES STUDY. *Int J Cardiol.* 2017;241:223-8. DOI: 10.1016/j.ijcard.2017.04.005
Journal Impact Factor (2017): 4.034. (Q1) Cardiology and Cardiovascular Medicine. Rank: 78/369.

2. **Arenas de Larriva AP**, Alonso A, Norby FL, Roetker NS, Folsom AR.
CIRCULATING CERULOPLASMIN, CERULOPLASMIN-ASSOCIATED GENES, AND THE INCIDENCE OF VENOUS THROMBOEMBOLISM IN THE ATHEROSCLEROSIS RISK IN COMMUNITIES STUDY. *Journal of Thrombosis and Haemostasis.* 2019;17(5):818-26. DOI: 10.1111/jth.14420
Journal Impact Factor (2019): 4.157. (Q1) Hematology. Rank: 14/134

3. **Arenas de Larriva AP**, Limia-Perez L, Alcala-Diaz JF, Alonso A, Lopez Miranda J, Delgado-Lista J. CERULOPLASMIN AND CORONARY HEART DISEASE-A SYSTEMATIC REVIEW. *Nutrients.* 2020;12(10).
DOI: 10.3390/nu12103219.
Journal Impact Factor (2020): 4.546. (Q1) Nutrition and Dietetics. Rank: 14/124

A. Circulating ceruloplasmin, ceruloplasmin-associated genes, and the incidence of atrial fibrillation in the Atherosclerosis Risk in Communities study

Arenas de Larriva AP, Norby FL, Chen LY, Soliman EZ, Hoogeveen RC, Arking DE, Loehr LR, Alonso A.

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Circulating ceruloplasmin, ceruloplasmin-associated genes, and the incidence of atrial fibrillation in the atherosclerosis risk in communities study

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ABSTRACT

Background: Ceruloplasmin (CP) may promote structural changes in the atrium making it more arrhythmogenic. We assessed the associations between CP, CP-associated genetic variants, and incident atrial fibrillation (AF) in the Atherosclerosis Risk in Communities (ARIC) study.

Methods and results: We studied 10,059 men and women without prevalent AF aged 53 to 75 years in 1996–1998 and followed through 2012. Circulating CP was measured in stored blood samples obtained in 1996–1998. Polymorphisms rs11708215 and rs13072552, previously associated with CP concentrations, were measured in 10,059 and 8829 participants respectively. AF was ascertained from study electrocardiograms, hospital discharge codes, and death certificates. Multivariable Cox models were run to study the association between circulating CP, CP-associated polymorphisms, and the incidence of AF. Over 10.5 years of mean follow-up, 1357 cases of AF were identified. After adjusting for traditional risk factors and biomarkers, higher levels of circulating CP were associated with incident AF (hazard ratio [HR] 1.33, 95% confidence interval [CI] 1.11, 1.61 comparing top to bottom quartiles). Both rs11708215 and rs13072552 were significantly associated with CP levels. Presence of the CP-increasing alleles in rs11708215 and rs13072552, however, were significantly associated with lower risk of AF in whites (HR 0.84, 95%CI 0.76, 0.94, $p = 0.002$ and HR 0.83; 95%CI 0.69, 0.99, $p = 0.043$ respectively per CP-increasing allele in the final adjusted model) but not in African Americans.

Conclusions: Even though higher CP concentrations were associated with increased AF risk, genetic variants associated with higher CP decreased the risk of AF in whites. Our results suggest that circulating CP levels may not be causally related to risk of incident AF.

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1. Introduction

Atrial fibrillation (AF) is the most common clinically-significant arrhythmia worldwide. It is estimated that, in the United States alone, the number of people who suffer AF is approximately 2.5 million, with men 1.5 times as likely to be affected compared to women [1]. Despite the decline in morbidity and mortality from cardiovascular disease due to advances in prevention and treatment, AF has not followed a similar trend, and the incidence of AF is expected to increase [2].

Ceruloplasmin (CP) is an enzyme synthesized in the liver that is responsible for transport of circulating copper and is also involved in iron metabolism. It is an acute-phase reactant that may have antioxidant actions, but can also participate in the generation of free radicals that seem to underlie several illnesses such as myocardial infarction, arteriosclerosis, unstable angina, abdominal aortic aneurysm, vasculitis and peripheral arterial disease, and even dementia [3–6].

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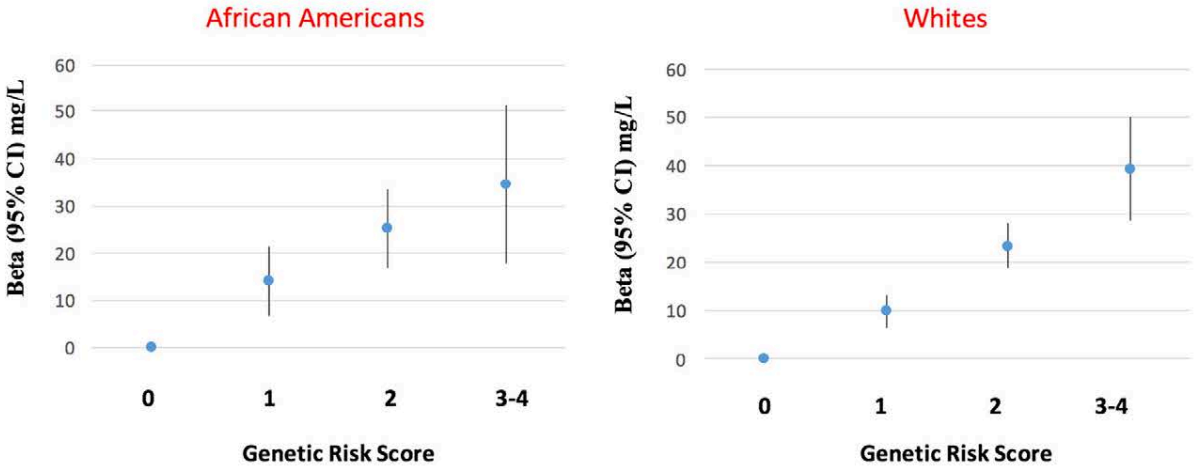

Fig. 1. Difference in CP concentration by number of CP-increasing alleles in rs11708215 and rs13072552, ARIC study, 1996–1998.

CP appears to promote structural changes in the atrium making it more arrhythmogenic. If this relationship between AF and CP is confirmed, new prevention approaches could be researched and we could identify individuals at increased risk of AF [7].

A recently published study showed that higher concentrations of CP in blood were associated with increased AF risk. In this same study, a variant of rs11708215, a single nucleotide polymorphism (SNP) located in the CP gene promoter, was associated with both higher CP concentrations in blood and increased AF risk [7]. These results, however, have not been replicated in other studies. Another SNP, rs13072552, also in the CP gene, has been associated with CP plasma concentration. This SNP was selected based on a GWAS in the Atherosclerosis Risk in Communities (ARIC) Study [4].

We addressed the association between rs11708215 and rs13072552, circulating CP and AF incidence in the ARIC Study. We hypothesized that higher concentrations of circulating CP would be associated with AF incidence and, following a Mendelian randomization framework, that if the association between circulating CP and AF incidence is causal then genetic variants associated with higher circulating CP would also increase the risk of AF.

2. Methods

2.1. Study population

The ARIC study is a community-based population study designed to investigate the causes of atherosclerosis and its clinical outcomes, as well as variation in cardiovascular risk factors, medical care, and disease by race and sex [8]. From 1987 to 1989 (ARIC study baseline), 15,792 adults (55.2% women; age, 45–64 years) from 4 US communities (Washington County, MD; suburbs of Minneapolis, MN; Jackson, MS; and Forsyth County, NC) were enrolled and underwent a home interview and clinic visit. Additional examinations were conducted in 1990 to 1992, 1993 to 1995, 1996 to 1998, and 2011 to 2013. Participants were mostly white in the Washington County and Minneapolis sites, exclusively black in Jackson, and a mix of both races in Forsyth County. Of the 11,656 participants in visit 4 (1996–1998), 11,484 had CP data available. Individuals with prevalent AF ($N = 524$) at visit 4 and those with missing data for CP ($N = 166$), missing information on rs11708215 ($N = 367$) or any other variable used in the statistical models ($N = 473$) were excluded from the study. We additionally excluded individuals who were not white or African American and any African American participants at the Minnesota and Washington County field centers because of small enrollment numbers ($N = 67$). After all exclusions, 10,059 participants remained and were included in this analysis. Medical history, demographic data, anthropometric data, blood pressure measurements, and fasting lipid assessments were obtained during visit 4 at the same time as the blood draw for CP measurement. The ARIC study has been approved by the Institutional Review Board at the University of Minnesota, Johns Hopkins University, Wake Forest University, University of North Carolina, Baylor College of Medicine, University of Texas Health Sciences Center at Houston, and University of Mississippi Medical Center. Participants provided written informed consent.

2.2. Ascertainment of AF

AF cases were identified from study visit ECGs, death certificates and by review of hospital discharge records [9,10]. At each study examination, a standard supine 12-lead resting ECG was recorded with a MAC PC Personal Cardiograph (Marquette Electronics, Milwaukee, WI) and transmitted to the ARIC ECG Reading Center (Epidemiological Cardiology Research Center, Wake Forest School of Medicine, Winston Salem, NC) for automatic coding. A cardiologist visually confirmed all AF cases automatically detected from the study ECGs. Information on hospitalizations during follow-up was obtained from annual follow-up calls and surveillance of local hospitals, with hospital discharge diagnosis codes collected by trained abstractors. AF during follow-up was defined as *International Classification of Disease, 9th Revision* (*ICD-9*), *Clinical Modification* diagnostic codes 427.31 or 427.32. AF cases detected in the same hospitalization with open cardiac surgery were not counted as cases. AF cases were also identified if ICD-9 code 427.3 or *International Classification of Diseases, 10th Revision* (ICD-10) code I48 was listed as a cause of death. A participant was considered to have prevalent AF at visit 4 (baseline for this analysis) if he or

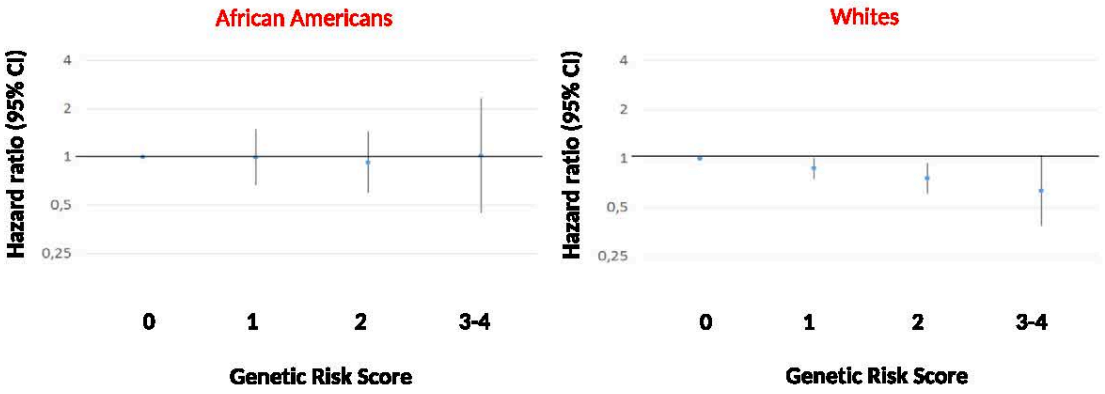

Fig. 2. Atrial fibrillation risk by number of CP-increasing alleles in rs11708215 and rs13072552, ARIC study, 1996–2012.

she had a prior AF hospitalization or had AF diagnosed through any of the study ECGs. In this analysis, the AF incident date was defined as the date of the first ECG showing AF (4% of our AF cases), the first hospital discharge with AF coded (96% of AF cases), or when AF was listed as a cause of death (0.1% of cases), whichever occurred earlier.

2.3. Covariates

At each study visit, participants underwent a physical exam, provided blood samples, and answered questionnaires. For the present analysis, information on all covariates was obtained at visit 4, with the exception of education, which was only assessed at baseline. Sex, race, date of birth, education, smoking, and alcohol use were self-reported by the study participant. Self-reported educational achievement, a surrogate measure of socioeconomic status, was categorized into 3 levels: less than high school, high school graduate, and greater than high school. Smoking status was categorized as never, former or current based on self-report. Alcohol consumption was ascertained by means of an interviewer-administered dietary questionnaire, and classified into three alcohol-use groups: never, former, or current [11]. Weight and height were measured with the participant wearing light clothing. Body mass index (BMI) was calculated as weight (in kilograms) divided by height squared (in meters). Blood pressure was measured twice and were averaged to define systolic and diastolic blood pressure. Hypertension was defined as a systolic blood pressure of ≥140 mm Hg or a diastolic pressure of ≥90 mm Hg or use of antihypertensive medication. Diabetes was defined as a fasting blood glucose ≥126 mg/dL, a non-fasting blood glucose >200 mg/dL, a self-reported physician diagnosis of diabetes, or use of antidiabetic medication [12].

Prevalent heart failure (HF) was identified by the Gothenburg criteria [13] or self-report of HF medication use in the past 2 weeks at the baseline visit. During follow-up, prevalent HF at each visit was identified as having a hospitalization with an ICD-9 code 428.0 during follow-up prior to that exam [14]. Myocardial infarction was based on self-report at visit 1 and adjudicated events between visit 1 and visit 4 [15]. History of stroke was defined as an adjudicated definite or probable hospitalized stroke occurring in a participant prior to visit 4 or a history of physician-diagnosed stroke at the baseline interview [16,17].

2.4. Biomarker assays and genotyping

Plasma CP concentrations were measured in 2010–2011 from Visit 4 plasma samples (stored at −70 °C since collection in 1996–1998) by immunoturbidimetric assay using an automated chemistry analyzer (Olympus AU400e, manufacturer Olympus Life Science Research Europa GmbH). The CP turbidimetric procedure was calibrated every 14 days by using Olympus Serum Protein Multi-calibrator 2 (Cat #ODR3023), which was traceable to IFCC International Reference Preparation CRM470 (RPPHS). The inter-assay coefficient of variation for CP was 6.8%. Alanine transaminase (ALT), aspartate aminotransferase (AST), and gamma-glutamyltransferase (GGT) were simultaneously measured in Visit 4 plasma samples using an Olympus AU400e automated chemistry analyzer (manufacturer Olympus Life Science Research Europa GmbH) according to the manufacturer's protocol. Inter-assay coefficients of variation were 11.1% for ALT, 8.5% for AST and 9.3% for GGT [18].

N-terminal pro–B-type natriuretic peptide (NT-proBNP) was measured by using an electrochemiluminescent immunoassay on an automated Cobas e411analyzer (Roche Diagnostics, Indianapolis, IN) with lower limit of detection ≤5 pg/mL and coefficient of variation 3.5 to 4.7% [16]. High sensitivity C-reactive protein (hs-CRP) levels were measured by using an immunonephelometric assay on a BNII autoanalyzer (Siemens Healthcare Diagnostics, Deerfield, IL) with a reliability coefficient of 0.9. Cardiac troponin T (cTnT) levels were measured by using a novel precommercial highly sensitive assay, Elecsys Troponin T (Roche Diagnostics), on an automated Cobas e411 analyzer with a lower limit of detection of 0.003 μg/L.

The rs11708215 SNP was genotyped using the Sequenom iPLEX assay, while rs13072552 was genotyped with the Affymetrix Genome-Wide Human SNP Array 6.0.

2.5. Statistical analysis

Baseline characteristics of the overall population were tabulated by CP quartiles. We report means for continuous variables and counts with percentages for categorical variables.

Cox proportional hazards models were used to determine the association between CP concentrations and incident AF. Follow-up time was calculated from the date of visit 4 to the incidence of AF, death, lost to follow-up, or December 31, 2012, whichever occurred first. Separate analyses were performed with circulating CP categorized into quartiles and as a continuous variable (in standard deviation units). The following models with incremental adjustments were used to analyze the CP-AF association: model 1: adjustment for age, sex, race, and ARIC study site; model 2: model 1 plus adjustment for BMI, height, alcohol drinking, smoking status, diabetes mellitus, educational level, systolic and diastolic blood pressure, total cholesterol and its fractions, liver enzymes and use of medications (antihypertensive and corticosteroids); model 3: model 2 plus history of heart failure, MI, and stroke; and model 4: model 3 plus biomarkers CRP, NT-proBNP and troponin. We conducted a sensitivity analysis excluding cases identified during the first 2 years of follow-up to avoid reverse causation (undiagnosed AF increasing circulating CP).

Secondly, race-specific linear regression models were used to test the association between *CP* gene SNPs (rs11708215, rs13072552) and CP concentrations. Analyses in African Americans were adjusted for the first 10 genetic principal components to correct for population stratification.

Thirdly, a race-specific Cox model was used to test associations between *CP* gene SNPs rs11708215 and rs13072552 separately, and AF risk, adjusting for age, sex, and ARIC study site and for covariates listed above in Models 2–4 as well as for CP concentrations to test whether any association with rs11708215 or rs13072552 was mediated by concentrations of circulating CP. We explored the associations using additive genetic models and estimating the associations by specific genotypes, using homozygous for the allele associated with lower CP as reference. We also used a 2-stage least square model to estimate the effect of circulating ceruloplasmin on AF risk using the two SNPs as instrumental variables.

Finally, we created a CP-related genetic risk score (GRS) adding up the number of CP-increasing alleles in the two SNPs (rs11708215 and rs13072552) (range, 0–4) and also categorized the population by haplotypes formed by combinations of the 2 SNPs, studying the association of GRS and *CP* gene haplotypes with CP concentrations and the incidence of AF in multivariable race-specific models. We used an unweighted genetic score since there are no large studies that could provide validated weights.

3. Results

After the exclusion of the participants listed above, this analysis included 10,059 (mean age 62.7 ± 5.6 years, 21.9% African American and 56.7% female). Mean CP levels were higher in African Americans than whites (311.6 ± 71.1 mg/L versus 296.8 ± 78.3 mg/L). The baseline demographic characteristics stratified by CP quartiles for the overall population are shown in Table 1. Overall, those with higher concentrations of circulating CP were more likely to be women, African American, had higher concentrations of hsCRP and NT-proBNP, but lower concentrations of cTnT.

3.1. Associations of CP concentration with incident AF

Table 2 presents the associations between circulating CP, stratified by quartiles and as a continuous variable, and AF risk. During a mean follow-up of 10.5 years, a total of 1357 individuals developed AF (212 AF events in African American and 1145 in whites). Higher levels of circulating CP were associated with incident AF in all the adjusted models. Individuals with CP in the highest quartile had significantly higher risk for AF than those in the lowest quartile (HR 1.38, 95%CI 1.08, 1.55) after adjusting for traditional risk factors. The association was comparable in the fully adjusted model including other biomarkers (HR 1.33, 95% CI 1.11, 1.61). A similar direct association was observed when we modeled circulating CP as a continuous variable (HR 1.06, 95%CI 0.99, 1.13, per 1-standard deviation difference in CP). Results were essentially unchanged after excluding 33 events occurring in the first 2 years of follow-up.

3.2. Association between rs11708215, rs13072552 and CP concentration

We performed a race-stratified analysis between CP concentration and the SNPs rs11708215 and rs13072552 located in or near the *CP* gene in chromosome 3 in 10,059 and 8829 subjects respectively (Table 3). The CP-increasing alleles frequency was different in whites and African Americans. A higher number of CP-increasing alleles in both SNPs was associated with higher concentrations of CP: 14.4 (95%CI 8.8, 20.1; $p < 0.001$) and 13.3 (95%CI 10.9, 15.8, $p < 0.001$) mg/L in African Americans and whites respectively for rs11708215, with the corresponding results being 5.9 (95%CI 1.7, 10.1; $p = 0.006$) and 21.6 (95%CI 17.6, 25.6; $p < 0.001$) mg/L for rs13072552. The R-squared for the association of each SNP with circulating CP ranged between 0.006 and 0.012.

3.3. Association between rs11708215, rs13072552 and AF risk

We next investigated the relation between rs11708215, rs13072552 and incidence of AF separately in whites and African Americans (Table 4). Contrary to our initial hypothesis, presence of the CP-increasing alleles in rs11708215 and rs13072552 were significantly associated with lower risk of AF in whites (HR 0.84, 95%CI 0.76, 0.94, $p = 0.002$ and HR 0.83, 95%CI 0.69, 0.99, $p = 0.04$ respectively for each CP-increasing allele in the final adjusted model) but not in African

Table 1
Baseline characteristics of the overall population by ceruloplasmin (CP) quartiles, ARIC study, 1996–1998.

CP mg/L	Quartile 1 <248.6	Quartile 2 248.6 to <285.3	Quartile 3 285.3 to <336.8	Quartile 4 ≥336.8
N	2517	2515	2513	2514
Age	63.1 ± 5.6	63.2 ± 5.6	62.8 ± 5.6	61.8 ± 5.5
Gender (% women)	21	45	68	92
African American (%)	13	20	28	26
BMI (kg/m^2)	28.8 ± 5.0	28.7 ± 5.3	29.1 ± 5.9	28.3 ± 5.9
Height (cm)	172.6 ± 8.8	169.1 ± 9.4	165.8 ± 8.9	162.7 ± 6.9
Diabetes mellitus (%)	17	17	17	12
HTN medication (%)	42	41	43	44
SBP (mmHg)	126 ± 18	127 ± 18.9	129 ± 19.4	127 ± 19.3
DBP (mmHg)	71 ± 9.8	71 ± 10.2	71 ± 10.6	70 ± 10.3
Smoker, current (%)	22	25	26	27
Alcohol, current (%)	27	26	23	24
Total cholesterol (mg/dL)	191 ± 34.2	200.2 ± 34.6	205.3 ± 37.3	206 ± 36.8
LDL-c (mg/dL)	119.4 ± 31.2	125.4 ± 31.8	127.7 ± 34.6	118.4 ± 35.1
HDL-c (mg/dL)	43.8 ± 13.4	47.7 ± 14.7	50.7 ± 15.7	59.3 ± 17.6
Triglycerides (mg/dL)	138.5 ± 69.1	135.2 ± 66.6	134 ± 65.8	140.8 ± 66.8
hs-cTnT (μg/L)	0.9 ± 2.9	0.7 ± 0.8	0.7 ± 1.4	0.5 ± 0.8
hsCRP (mg/L)	3.5 ± 7.2	3.3 ± 4.7	4.2 ± 5.2	6.6 ± 7.9
NT-proBNP (pg/mL)	127.2 ± 525	131.1 ± 470.6	132.1 ± 524.4	141.7 ± 244.6
GGT (U/L)	29 ± 26.3	30.7 ± 39.9	31 ± 35.8	29.5 ± 44.9
AST (U/L)	24 ± 14.7	24.1 ± 13.8	24.1 ± 11.2	23.1 ± 10.5
ALT (U/L)	20.2 ± 13.7	19.5 ± 13	19 ± 11.3	16.7 ± 9.1

Values correspond to means or percent. Plus-minus values are means ± SD.
BMI, body mass index; HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-cTnT, high-sensitivity cardiac troponin T; hs-CRP, high-sensitivity C-reactive protein; NT-proBNP, N-terminal pro–B-type natriuretic peptide. GGT, γ glutamyl transpeptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Americans (corresponding HRs 0.92, 95% CI 0.67, 1.25, $p = 0.58$ and 1.01, 95% CI 0.81, 1.26, $p = 0.92$). Additional adjustment for polymorphisms rs11708215 and rs13072552 did not affect the association between circulating ceruloplasmin and AF incidence (data not shown). Results from a two-stage least squares regression analysis with the two polymorphisms as instrumental variables were consistent with these results, showing an inverse association of genetically-determined CP with AF risk in whites but not in African Americans (Table 5).

3.4. Difference in CP concentration and AF risk by number of risk alleles and haplotypes in rs11708215 and rs13072552

The CP-related GRS showed a linear direct association with circulating CP. Participants with 3 or 4 CP-increasing alleles had the highest blood CP concentration. This difference was significant in both African Americans (Beta 34.5, 95%CI 18, 51 mg/L) and whites (Beta 36.3, 95%CI, 28.6, 49.9 mg/L) (Fig. 1). In contrast, we did not find any significant association between GRS and increased AF risk in African Americans and a potentially lower risk of AF with higher GRS in whites (Fig. 2). Similar results were obtained when participants were categorized according to haplotypes in both SNPs.

4. Discussion

Our study is the largest prospective study to date showing that higher concentrations of circulating CP, an inflammatory plasma protein, are associated with AF. Consistent with previous observations, we found that variants in SNPs rs11708215 and rs13072552, which are in or near the *CP* gene in chromosome 3, were associated with circulating concentrations of CP in a biracial cohort. In contrast, effect alleles associated with higher CP concentrations in these 2 alleles were not associated with higher incidence of AF. In fact, and contrary to our initial hypothesis, variants associated with *higher* CP concentrations were associated with *lower* risk of AF in whites. These findings do not support a direct causal role of CP on AF risk, though statistical power is possibly limited.

A previous publication from the Malmö Preventive Project in southern Sweden, including 3900 participants, described an association between CP concentrations and AF incidence [7]. Our study confirms and

Table 2
Association between CP concentration and AF risk, ARIC study, 1996–2012.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	Continuous[a]	*P*-value^
CP (mg/L)	≤248.6	248.6 to <285.3	285.3 to <336.8	≥336.8	CP/σ	
# AF cases	359	362	335	301	1357	
N	2514	2503	2492	2550	10,059	
	Hazard ratios (95% confidence intervals)					
Model 1	1 (ref.)	1.11 (0.96, 1.29)	1.18 (1.01, 1.39)	1.30 (1.08, 1.55)	1.04 (0.98, 1.11)	0.18
Model 2	1 (ref.)	1.13 (0.98, 1.32)	1.21 (1.03, 1.42)	1.38 (1.15, 1.66)	1.07 (1.00, 1.14)	0.05
Model 3	1 (ref.)	1.12 (0.96, 1.30)	1.19 (1.02, 1.40)	1.37 (1.14, 1.65)	1.06 (0.99, 1.14)	0.06
Model 4	1 (ref.)	1.12 (0.97, 1.30)	1.19 (1.01, 1.40)	1.33 (1.11, 1.61)	1.06 (0.99, 1.13)	0.1

Model 1: adjustment for age, sex, race, and ARIC study site.
Model 2: Model 1 + adjustment for BMI, height, alcohol drinking, diabetes mellitus, educational level, smoking status, systolic and diastolic blood pressure, total cholesterol and its fractions, liver enzymes and use of medications (antihypertensive and corticosteroids).
Model 3: Model 2 + history of heart failure, MI, and stroke.
Model 4: Model 3 + biomarkers CRP, BNP and troponin.
[a] Per 1-standard deviation increase in CP. σ = Standard deviation = 77.07 mg/L.
^ *P*-value for the continuous analysis.

Table 3
Difference in ceruloplasmin concentration by rs11708215 and rs13072552 genotype by race, ARIC study, 1996–1998.

rs11708215					
	AA	AG	GG	Additive model	*P*-value^
African Americans ($N = 2205$)	1621	527	57	2205	
CP mean values (mg/L)	307.4	322.4	330.7	311.6	
Model 1	Ref.	14.6 (7.7, 21.6)	25.5 (6.9, 44.1)	14.0 (8.2, 19.8)	<0.001
Model 2	Ref.	15.2 (8.4, 22.0)	26.0 (8.0, 44.1)	14.4 (8.8, 20.1)	<0.001
Whites ($N = 7854$)	5008	2540	306	7854	
CP mean values (mg/L)	291.4	303.8	327.6	296.8	
Model 1	Ref.	12.9 (9.8, 16.1)	32.0 (24.4, 36.9)	14.2 (11.6, 16.7)	<0.001
Model 2	Ref.	11.9 (8.9, 14.9)	31.0 (23.8, 38.2)	13.3 (10.9, 15.8)	<0.001
rs13072552					
	GG	GT	TT	Additive model	*P*-value^
African Americans ($N = 1925$)	628	958	339	1925	
CP mean values (mg/L)	308.8	309.6	323.6	311.8	
Model 1	Ref.	1.3 (−5.3, 7.9)	11.8 (2.9, 20.6)	5.2 (0.8, 9.5)	0.02
Model 2	Ref.	1.1 (−5.4, 7.6)	13.6 (4.9, 22.2)	5.9 (1.7, 10.1)	0.006
Whites ($N = 6904$)	5941	921	42	6904	
CP mean values (mg/L)	294.1	314.4	341.5	297.1	
Model 1	Ref.	20.9 (16.4, 25.6)	52.9 (32.9, 72.9)	21.9 (17.7, 26.1)	<0.001
Model 2	Ref.	20.7 (16.4, 25.1)	51.8 (32.8, 70.7)	21.6 (17.6, 25.6)	<0.001

Measure of association: Beta coefficient (95% confidence intervals).
Model 1: adjustment for age, sex, ARIC study site and GWAS PCs.
Model 2: Model 1 + adjustment for BMI, height, alcohol drinking, diabetes mellitus, educational level, smoking status, systolic and diastolic blood pressure, total cholesterol and its fractions, liver enzymes, use of medications (antihypertensive and corticosteroids), history or heart failure, MI, stroke and biomarkers like CRP, BNP and troponin.
^ *P*-value for the additive model.

extends these findings to the large, middle-aged, biracial cohort of men and women in the ARIC study. After adjusting for traditional risk factors and different biomarkers, higher levels of circulating CP were associated with incident AF. In contrast to our results, the Malmö Preventive Project analysis described a higher risk of AF associated with the CP-increasing allele in SNP rs11708215. This discrepancy may be due to differences in sociodemographic and clinical characteristics of the study populations, diverse sample sizes, and heterogeneity in AF ascertainment method.

CP has been reported to possess both oxidative and anti-oxidative functions. It has been seen that the overproduction of reactive oxygen species (ROS) is associated with both AF and CP pathogenicity [19]. When this occurs, the body's antioxidant systems such as catalase, superoxide dismutase and glutathione are saturated and unable to

Table 4
Association of rs11708215 and rs13072552 genotype with incidence of atrial fibrillation by race, ARIC study, 1996–2012.

rs11708215					
	AA	AG	GG	Additive model	*P*-value^
African Americans ($N = 2205$)	1621	527	57	2205	
AF cases	159	46	7	212	
Model 1	1 (ref.)	0.87 (0.62, 1.20)	1.23 (0.58, 2.63)	0.90 (0.66, 1.21)	0.47
Model 2	1 (ref.)	0.89 (0.64, 1.24)	1.39 (0.65, 2.99)	0.92 (0.68, 1.26)	0.61
Model 3	1 (ref.)	0.88 (0.63, 1.23)	1.38 (0.64, 2.96)	0.92 (0.67, 1.25)	0.58
Whites ($N = 7854$)	5008	2540	306	7854	
AF cases	766	339	40	1145	
Model 1	1 (ref.)	0.84 (0.74, 0.96)	0.82 (0.60, 1.13)	0.87 (0.77, 0.96)	0.008
Model 2	1 (ref.)	0.84 (0.73, 0.95)	0.77 (0.56, 1.05)	0.85 (0.77, 0.95)	0.003
Model 3	1 (ref.)	0.83 (0.73, 0.94)	0.75 (0.54, 1.03)	0.84 (0.76, 0.94)	0.002
rs13072552					
	GG	GT	TT	Additive model	*P*-value^
African Americans ($N = 1925$)	628	958	339	1925	
AF cases	54	92	33	179	
Model 1	1 (ref.)	0.98 (0.73, 1.31)	0.99 (0.66, 1.47)	1.08 (0.87, 1.34)	0.47
Model 2	1 (ref.)	0.89 (0.66, 1.20)	0.92 (0.61, 1.38)	1.01 (0.81, 1.27)	0.91
Model 3	1 (ref.)	0.89 (0.66, 1.20)	0.91 (0.61,1.37)	1.01 (0.81, 1.26)	0.92
Whites ($N = 6904$)	5941	921	42	6904	
AF cases	883	123	4	1010	
Model 1	1 (ref.)	0.91 (0.75, 1.10)	0.62 (0.23, 1.66)	0.89 (0.75, 1.06)	0.19
Model 2	1 (ref.)	0.86 (0.71, 1.03)	0.68 (0.25, 1.82)	0.85 (0.71, 1.02)	0.08
Model 3	1 (ref.)	0.84 (0.69, 1.02)	0.65 (0.24, 1.75)	0.83 (0.69, 0.99)	0.04

Measure of association: Hazard ratio (95% confidence intervals).
Model 1: adjustment for age, sex and ARIC study site and GWAS PCs (in African Americans).
Model 2: Model 1 + adjustment for BMI, height, alcohol drinking, diabetes mellitus, educational level, smoking status, systolic and diastolic blood pressure, total cholesterol and its fractions, liver enzymes, use of medications (antihypertensive and corticosteroids), history or heart failure, MI, stroke and biomarkers like CRP, BNP and troponin.
Model 3: Model 2 + CP concentration.
^ *P*-value for the additive model.

Table 5
Results from two-stage least squares regression analysis with rs11708215 and rs13072552 as instruments, circulating ceruloplasmin as the main independent variable, and AF risk as the outcome, ARIC study, 1996–2012.

	rs11708215		rs13072552	
	Whites	African Americans	Whites	African Americans
B coefficients	−0.001	0.001	−0.001	0.001
P-value	0.039	0.726	0.138	0.731

counteract oxidative stress, leading to tissue damage. CP is capable of promoting the activation of the NO oxidase and consume NO catalytically reducing its bioavailability in plasma. Animal studies have shown that NO and NO synthases play a key role in the normal cardiac physiology [20,21]. NO is a cardioprotective agent due to, among other mechanisms, inhibition of oxidative stress. Therefore, high levels of CP in the body can stimulate the activity of NO oxidase causing a decrease of NO and tilting the balance towards an oxidative role. This oxidative stress may produce cardiac electrical activity alterations, damaging ion channels and finally leading to the AF development [22,23].

Our study has several potential clinical implications. First, since patients with high levels of CP are more likely to develop AF, information on CP concentrations may facilitate efforts to identify high-risk individuals. And, second, future studies could determine whether CP measurements can be used to monitor the efficacy of interventions aimed to prevent AF.

4.1. Limitations

Although hospital discharge codes being used for identifying incident AF cases have shown to be valid [9], AF cases managed exclusively in outpatient settings and those who are asymptomatic are certainly missed. In addition, there may be some misclassification of the CP concentrations exposure since there is no follow-up information on circulating CP after visit 4. As a result, if the CP measures happened to change over time, there is no additional information to examine such changes from follow-up data. Finally, our Mendelian randomization analysis is limited due to the weak association between the CP-related variants and circulating CP and to the potential association of CP-related variants with other cardiovascular risk factors (pleiotropy).

5. Conclusion

High levels of circulating CP are associated with increased incidence of AF. The two SNPs studied were associated with increased CP levels in both whites and African Americans. Opposite to our initial hypothesis, the presence of the effect allele in rs11708215 SNP was associated with significantly lower risk of AF in whites, but not in African Americans. Our results suggest that CP can be one of many inflammatory intermediaries involved in the development of AF. Therefore, additional studies are needed to understand the causal mechanism behind this association to advance our ability to prevent this common arrhythmia.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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B. Circulating ceruloplasmin, ceruloplasmin-associated genes and the incidence of venous thromboembolism in the Atherosclerosis Risk in Communities study

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ORIGINAL ARTICLE

Circulating ceruloplasmin, ceruloplasmin-associated genes and the incidence of venous thromboembolism in the Atherosclerosis Risk in Communities study

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Essentials

- Ceruloplasmin (CP) is an acute-phase reactant and a potential biomarker of atherothrombotic risk.
- We assessed associations between CP and venous thromboembolism (VTE) risk in 9933 individuals.
- Higher circulating CP but not CP-related genes were associated with greater incident VTE rates.
- Circulating CP could be considered a non-causal biomarker of VTE risk in the community.

Summary. *Background:* Ceruloplasmin (CP) is an acute-phase reactant and a potential biomarker of atherothrombotic risk. We assessed the associations between CP, CP-associated genetic variants and incident venous thromboembolism (VTE) in the Atherosclerosis Risk in Communities study. *Methods and results:* In an observational study, 9933 men and women aged 53–75 years without prevalent VTE were included in 1996–1998 and followed through 2011. Circulating CP was measured in stored blood samples obtained in 1996–1998. Polymorphisms rs11708215 and rs13072552, which have been previously associated with CP concentrations, were measured in 8439 participants. VTEs were identified from hospital discharge codes and validated by physician review of medical records and imaging reports. Over a mean of 10.5 years of follow-up, 376 cases of VTE were identified. The association between circulating CP, CP-associated polymorphisms and the incidence of VTE was estimated. After adjustment for traditional risk factors and biomarkers, higher concentrations of circulating CP were associated with greater incident VTE rates (hazard ratio 1.82, 95% confidence interval 1.12–2.95, comparing the 87.5–100th percentile with the bottom quartile). Both rs11708215 and rs13072552 were associated with CP concentrations but not with VTE risk. *Conclusions:* Even though high CP concentrations were associated with an increased VTE risk, CP-associated genetic variants were not associated with a higher risk of VTE. Our results suggest that circulating CP concentrations may not be causally related to the risk of incident VTE.

Keywords: ceruloplasmin; oxidative stress; single-nucleotide polymorphism; venous thromboembolism.

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Introduction

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are manifestations of venous thromboembolism (VTE), the third most common cardiovascular disease after myocardial infarction and stroke. [1,2].

It is estimated that at least 900 000 people are affected by VTE (1–2 per 1000 adults) each year in the USA. This results in 100 000 premature deaths [3,4], of which >10% will occur within 1 month of diagnosis. [1,2]. Because of the public health burden and expense associated with VTE, additional biomarkers are needed to help detect or predict VTE and/or monitor the treatments prescribed.

Ceruloplasmin (CP) is an enzyme synthesized in the liver that is responsible for the transport of circulating copper. CP is also involved in iron metabolism. It is an acute-phase reactant that may have antioxidant actions but can also participate in the generation of free radicals that may underlie several illnesses, such as myocardial infarction, arteriosclerosis, unstable angina, abdominal aortic aneurysm, vasculitis, peripheral arterial disease, and even dementia [5,6].

There is strong evidence that inflammation, during which CP levels are increased, is associated with an increased risk of atherothrombosis [7,8]. Several studies have demonstrated a relationship between various proteins involved in inflammatory processes and VTE. For example, the Atherosclerosis Risk in Communities (ARIC) study previously found that elevated high-sensitivity C-reactive protein (hsCRP), but not fibrinogen, was independently associated with an increased risk of VTE [9]. Inflammation seems to trigger a chain reaction whereby procoagulant factors are activated and the fibrinolytic pathway is inhibited [10]. However, Sveinsdottir *et al.* found no significant relationship between VTE incidence and CP or other inflammatory markers (fibrinogen, orosomucoid, α_1-antitrypsin, and haptoglobin) [11]. Additionally, two single-nucleotide polymorphisms (SNPs) located in the CP gene promoter are associated with CP concentrations in blood (rs11708215 and rs113072552) [12]. We addressed the association between these SNPs, circulating CP and VTE incidence in the ARIC study. We hypothesized that higher circulating CP concentrations would be associated with greater VTE incidence, and, following a Mendelian randomization framework, that if the association between circulating CP and VTE incidence is causal then genetic variants associated with higher circulating CP concentrations would also increase the risk of VTE.

Methods

Study population

The ARIC study is a community-based population study designed to investigate the causes of cardiovascular disease. From 1987 to 1989 (ARIC study baseline), 15 792 adults (55.2% women; age, 45–64 years) from four US communities (Washington County, MD; suburbs of Minneapolis, MN; Jackson, MS; and Forsyth County, NC) were enrolled and underwent a home interview and clinic visit. Additional examinations were conducted in 1990–1992, 1993–1995, 1996–1998, 2011–2013, and 2016–2017. Participants were mostly white in the Washington County and Minneapolis sites, exclusively African Americans in Jackson, and a mix of both in Forsyth County [13].

Of the 11 656 participants attending visit 4 (1996–1998), we excluded individuals with prevalent VTE at visit 1 ($N = 238$), with an incident VTE event before visit 4 ($N = 49$), with missing data for CP ($N = 153$), taking anticoagulants at visit 4 ($N = 235$), whose follow-up ended on the date of the visit 4 examination ($N = 2$), and with missing information for body mass index (BMI) ($N = 38$) or any other variable used in the statistical models ($N = 939$). We additionally excluded individuals who were not white or African American and any African American participants at the Minnesota and Washington County field centers, because of small enrollment numbers ($N = 69$).

Ascertainment of VTE

Staff contacted ARIC participants annually by telephone and asked about all hospitalizations in the previous year. In addition, the ARIC study conducted surveillance of hospital discharge lists from local hospitals, and obtained all International Classification of Diseases (ICD) discharge codes. For ICD codes indicating possible VTE events, staff obtained copies of the hospital records. To validate VTE events, two physicians reviewed the records by using standardized criteria, requiring positive imaging test findings for diagnosis of DVT and PE. We restricted DVTs for this analysis to those occurring in the lower extremities or vena cava, because upper-extremity DVTs were relatively few in number and almost always resulted from the use of venous catheters [14].

Covariates

At each study visit, participants underwent physical assessments, provided blood samples, and answered questionnaires. For the present analysis, information on all covariates was obtained at visit 4, with the exception of education, which was assessed only at baseline. Sex, race, date of birth and hormone replacement therapy (HRT) were self-reported by the study participant. Weight and height were measured with the participant wearing light clothing. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Blood pressure was measured twice and averaged to define systolic and diastolic blood pressure. Hypertension was defined as a systolic blood pressure of ≥ 140 mmHg, a diastolic blood pressure of ≥ 90 mmHg, or the use of antihypertensive medication. Diabetes was defined as a fasting blood glucose level of ≥ 126 mg dL^{-1}, a non-fasting blood glucose level of > 200 mg dL^{-1}, a self-reported physician diagnosis of diabetes, or the use of antidiabetic medication.

Biomarker assays and genotyping

Plasma CP concentrations were measured in 2010–2011 from visit 4 plasma samples (stored at − 70 °C since collection in 1996–1998) with an immunoturbidimetric assay by use of an automated chemistry analyzer (Olympus AU400e; Olympus Life Science Research Europa, München, Germany). The CP turbidimetric procedure was calibrated every 14 days by the use of Olympus

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Serum Protein Multi-calibrator 2 (Cat. no. ODR3023), which was traceable to International Federation for Clinical Chemistry International Reference Preparation CRM470 (RPPHS). The interassay coefficient of variation for CP was 6.8%.

Levels of hsCRP were measured with an immunonephelometric assay on a BNII autoanalyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA), with a reliability coefficient of 0.9.

The rs11708215 SNP was genotyped with the Sequenom iPLEX assay, and the rs13072552 SNP was genotyped with the Affymetrix Genome-Wide Human SNP Array 6.0.

The ARIC study had previously exhausted most baseline citrate plasma samples. Therefore, in this analysis, we used D-dimer and factor XI concentrations measured in fasting citrate plasma collected at ARIC visit 3 (in 1993–1995) and stored unthawed at − 70 °C until analysis in 2014. The Laboratory for Clinical Biochemistry Research at the University of Vermont used an immunoturbidimetric assay (Liatest D-DI; Diagnostica Stago, Parsippany, NJ, USA) on the Evolution analyzer (Diagnostica Stago) for D-dimer, and sandwich ELISA with affinity-purified polyclonal antibodies from Affinity Biologicals (Ancaster, Ontario, Canada) for factor XI. The analytical coefficient of variation for the D-dimer assay was 4–16%. Blind analysis of 73 pairs of ARIC samples split at the time of blood draw and stored until 2014 yielded an intraclass reliability coefficient of 0.92 [15].

For FXI, the coefficient of variation for control samples during this study averaged 9.6%. Blind analysis of 74 pairs of ARIC samples split at the time of blood draw and stored until 2014 yielded an intraclass reliability coefficient of 0.81 [16].

Other hemostatic factors were measured at ARIC visit 1 (1987–1989). FVIII activity was measured by determining the ability of the tested sample to correct the clotting time of human FVIII-deficient plasma obtained from George King Biomedical, Overland Park, Kansas. von Willebrand factor (VWF) antigen was determined by the use of ELISA kits from American Bioproducts, Canton, MA. Activated partial thromboplastin time (APTT) was measured on an automated coagulometer (Coag-A-Mate X-2; General Diagnostics, Turbhe, Navi Mumbai, India). The reference material for assays was the Universal Coagulation Reference Plasma (Thromboscreen; Pacific Hemostasis, Curtin Matheson Scientific, Houston, TX, USA). Reliability coefficients (method variance plus intraindividual variance divided by total variance) obtained from repeated testing of individuals over a period of several weeks were 0.86 for FVIII, 0.68 for VWF, and 0.92 for APTT [17].

Statistical analysis

Cox proportional hazards models were used to estimate the association between CP concentrations and incident VTE. Time to follow-up was defined as the time between visit 4 and VTE occurrence, death, loss to follow-up, or the end of 2011, whichever occurred first. Initially, we explored the shape of the association of CP concentration with VTE risk by using restricted cubic splines, which allowed us to test for linearity. The circulating CP concentration was modeled by the use of quartiles (with the highest quartile split in two) and as a continuous variable (scaled per standard deviation increment).

The following incrementally adjusted models were used to analyze the CP–VTE association: model 1 – adjustment for age, sex, race, BMI, and current HRT; model 2 – model 1 plus adjustment for diabetes mellitus, systolic blood pressure, APTT, VWF, D-dimer, FVIII, FXI, and hsCRP. Other atherosclerotic risk factors, such as diastolic blood pressure, smoking, lipid levels, and physical activity, were not strong VTE risk factors in the ARIC study, and were therefore not examined. We also performed a sensitivity analysis with adjustment for participants' cancer status (yes or no), which had no material impact on the results.

In a second step, we used race-specific linear regression models testing the association between CP gene SNPs (rs11708215 and rs13072552) and CP concentration. Analyses in African Americans were adjusted for the first 10 principal components of ancestry (PCAs) to correct for population stratification.

Third, a race-specific Cox model was used to test associations of CP gene SNPs rs11708215 and rs13072552, separately, with VTE risk, with adjustment for age, sex, race, BMI, HRT, and PCAs (in African Americans), and for covariates listed above in model 2 as well as for CP concentration, to test whether any association between rs11708215 or rs13072552 might be mediated by circulating CP.

Finally, we created a CP-related genetic risk score (GRS) by summing the number of CP-increasing alleles in the two SNPs (rs11708215 and rs13072552) (range, 0–4), and also categorized the population into haplotypes formed by combinations of the two SNPs. We assessed the associations of GRS and CP gene haplotypes with CP concentration and the incidence of VTE in multivariable race-specific models. We used an unweighted GRS, because there are no large studies that could provide validated weights.

Results

This ARIC visit 4 sample included 9933 participants at risk for VTE (mean age, 62.7 ± 5.6 years; 20.1% African Americans and 55.7% females). Mean CP concentrations were higher in African Americans than in whites (311.9 ± 71.7 mg L^{-1} versus 296.1 ± 78.1 mg L^{-1}). The baseline demographic characteristics stratified by CP quartiles for the overall sample are shown in Table 1. Those with higher circulating CP concentrations were

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Table 1 Baseline characteristics (mean ± standard deviation or percentage) of the overall sample by ceruloplasmin (CP) quartiles in the Atherosclerosis Risk in Communities study, 1996–1998

	CP (mg L^{-1})			
Characteristic	Quartile 1 ≤ 248	Quartile 2 248 to < 284.6	Quartile 3 284.6 to < 335	Quartile 4 ≥ 335
N	2486	2487	2480	2480
Age (years)	63 ± 5.6	63 ± 5.7	63 ± 5.7	62 ± 5.5
Women (%)	20	44	67	91
African American (%)	12	18	27	25
BMI (kg m^{-2})	28.9 ± 4.9	28.6 ± 5.2	29.0 ± 5.8	28.2 ± 5.8
HRT use (%)*	3	5	11	42
Diabetes mellitus (%)	18	17	17	12
SBP (mmHg)	126 ± 17	127 ± 19	129 ± 19	127 ± 19
DBP (mmHg)	71 ± 9	71 ± 10	71 ± 10	70 ± 10
Smoker, current (%)	21	25	27	26
Total cholesterol (mg dL^{-1})	191 ± 35	200 ± 35	205 ± 38	206 ± 36
LDL cholesterol (mg dL^{-1})	119 ± 30	125 ± 31	127 ± 33	118 ± 34
HDL chosterol (mg dL^{-1})	44 ± 13	47 ± 14	50 ± 15	59 ± 17
Triglycerides (mg dL^{-1})	147 ± 98	140 ± 83	141 ± 86	146 ± 77
hsCRP (mg L^{-1})	3.6 ± 7.4	3.3 ± 4.7	4.2 ± 5.3	6.5 ± 7.6
APTT (s)†	29.4 ± 3.0	29.2 ± 2.9	29.1 ± 3.1	28.8 ± 2.8
Factor VIII (%)†	125 ± 37	127 ± 35	129 ± 36	129 ± 35
VWF (%)†	112 ± 43	114 ± 44	116 ± 47	113 ± 43
D-dimer (μg mL^{-1})‡	0.39 ± 1.05	0.46 ± 1.33	0.48 ± 1.13	0.62 ± 1.82
Factor XI (%)‡	107 ± 24	111 ± 25	115 ± 26	118 ± 28

APPT, activated partial thromboplastin time; BMI, body mass index; DBP, diastolic blood pressure; HRT, hormone replacement therapy; hsCRP, high-sensitivity C-reactive protein; SBP, systolic blood pressure; VWF, von Willebrand factor. *Women only. †Visit 1 value (1987–1989). ‡Visit 3 value (1993–1995). The rest of the covariates were measured at visit 4.

more likely to be women, to be African American, and to have higher hsCRP concentrations.

Associations of CP concentration with incident VTE

During a mean follow-up of 10.5 years, a total of 376 individuals developed VTE (110 events in African Americans and 266 in whites). Table 2 shows the associations between circulating CP concentration, stratified by quartiles (last quartile split in two) and as a continuous variable, and VTE risk. Higher circulating CP concentrations were associated with greater incidence of VTE in all of the adjusted models. Individuals with CP concentrations in the highest category (87.5–100th percentile) had 1.61-fold greater VTE risk than those in the lowest quartile (hazard ratio [HR] 1.61, 95% confidence interval [CI] 1.06–2.45) after adjustment for traditional risk factors. The association was comparable in the fully adjusted model including other biomarkers (HR 1.50, 95% CI 0.98–2.29). A similar association was observed when we modeled circulating CP concentration as a continuous variable in model 2 (HR 1.16, 95% CI 1.03–1.30 per one standard deviation increment in CP concentration). Associations between circulating CP concentrations and VTE risk were similar in whites (HR 1.16, 95% CI 1.01–1.34 per one standard deviation increment in CP concentration) and African Americans (HR 1.13, 95% CI 0.91–1.41). We also conducted stratified analysis by sex. HRs (95% CIs) for the association between CP concentration and VTE stratified by sex are shown in Tables S1 and S2. Higher CP concentrations were associated with an increased VTE risk in both men and women, without evidence of a significant interaction (*P* for interaction = 0.49). Given the more limited sample size in each analysis, estimates were more imprecise than in the combined analysis. Nevertheless, HRs for VTE in the top category were 1.5 in women and 1.6 in men.

One additional analysis was carried out with restriction of follow-up to the first 11 years. Associations were stronger than for the entire follow-up, indicating that there was some dilution of the effect over time (Table S3).

We also plotted the association between circulating CP concentration and VTE risk by modeling CP concentration with a restricted cubic spline. The risk increased up to the 87.5th percentile, and plateaued afterwards (Fig. 1).

Association between rs11708215, rs13072552, and CP concentration

We performed a race-stratified analysis between CP concentration and the SNPs rs11708215 and rs13072552 located in or near the CP gene in chromosome 3 in 8439 subjects (Table 3). The frequencies of CP-increasing alleles differed between whites and African Americans. For both SNPs, a higher number of CP-increasing alleles was associated with higher concentrations of CP: 30.3 mg L^{-1} (95% CI 11.5–49.1) and 29.8 mg L^{-1} (95% CI 22.–37.2)

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Table 2 Hazard ratios (95% confidence intervals) for the association between ceruloplasmin (CP) concentration and venous thomboembolism (VTE) risk; the Atherosclerosis Risk in Communities study, 1996–2011

	Quartile 1	Quartile 2	Quartile 3	75–87.5th percentile	87.5–100th percentile	Continuous*	*P*-value†
CP (mg L^{-1})	< 248	248 to < 284.6	284.6 to < 335	335 to < 386.4	≥ 386.4		
No. of VTE cases	87	92	85	60	52	376	
N	2486	2487	2480	1240	1240	9933	
Hazard ratios (95% confidence intervals)							
Model 1	1 (ref.)	1.09 (0.81–1.47)	0.99 (0.72–1.37)	1.53 (1.05–2.21)	1.61 (1.06–2.45)	1.20 (1.06–1.35)	0.003
Model 2	1 (ref.)	1.09 (0.81–1.46)	0.98 (0.71–1.36)	1.50 (1.03–2.18)	1.50 (0.98–2.29)	1.16 (1.03–1.30)	0.016

Model 1: adjustment for age, sex, race, body mass index (BMI), and hormone replacement therapy (HRT). Model 2: model 1 + adjustment for diabetes mellitus, systolic blood pressure, activated partial thromboplastin time, von Willebrand factor, D-dimer, factor VIII, factor XI, and high-sensitivity C-reactive protein. *Per one standard deviation increase in CP concentration (77.1 mg L−1). †*P*-value for the continuous analysis.

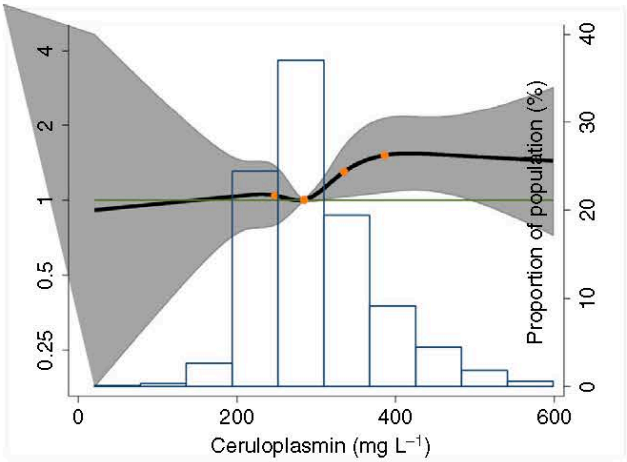

Fig. 1. Association of concentration of circulating ceruloplasmin with incidence of VTE presented as hazard ratio (solid line) and 95% confidence interval (shaded area) adjusted for age, sex, and race. The histogram represents the distribution of circulating ceruloplasmin in the study sample. Orange points corresponds to the values for the 25th, 50th, 75th and 87.5th percentiles of the ceruloplasmin distribution.

higher in African Americans and whites, respectively, for rs11708215, with the corresponding results being 13.6 mg L^{-1} (95% CI 4.6–22.6) and 53.8 mg L^{-1} (95% CI 34.9–72.7) higher for rs13072552 in the fully adjusted model. The proportion of variability in circulating CP concentration explained by these two SNPs was small (r^2 = 0.02). Reported differences in concentration reflect two risk alleles versus no risk alleles of the SNPs.

Association between rs11708215, rs13072552, and VTE risk

We next investigated the relationship of rs11708215 and rs13072552 with VTE incidence separately in whites and African Americans (Table 4). The presence of the CP-increasing alleles in rs11708215 and rs13072552 were not significantly associated with VTE risk in whites or African Americans.

Difference in CP concentration and VTE risk by number of risk alleles and haplotypes in rs11708215 and rs13072552

The CP-related GRS showed a linear association with circulating CP concentration (Table 5). Participants with three or four CP-increasing alleles had the highest blood CP concentrations. This difference was significant in both African Americans (Beta 11.6 mg L^{-1}, 95% CI 7.7–15.5) and whites (Beta 11.7 mg L^{-1}, 95% CI 9.8–13.6). In contrast, neither the GRS (Table 5) nor the haplotype categories (Table 6) were associated with VTE risk in whites. In contrast, there was some suggestion that African American with a GRS of 0 (Table 5) or haplotype AA/GG may have a lower VTE risk than those with other genotypes.

Discussion

This is the largest prospective study to date showing that a higher concentration of circulating CP, an inflammatory plasma protein, is associated with modestly increased VTE risk. We found that variants in SNPs rs11708215 and rs13072552, which are in or near the CP gene in chromosome 3, were associated with circulating CP concentrations in both whites and African Americans. In contrast, alleles in these two SNPs associated with higher CP concentrations were not associated with a greater VTE incidence. These findings do not support a direct

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Table 3 Association between rs11708215 and rs13072552 single-nucleotide polymorphism and difference in ceruloplasmin (CP) concentration

	AA	AG	GG
rs11708215			
African Americans (N = 1661)	1225	393	43
CP mean values (mg L^{-1})	307.4	322.4	330.7
Difference (mg dL^{-1}), model 1	Ref.	16.6 (9.5–23.7)	29.6 (10.7–48.5)
Difference (mg dL^{-1}), model 2	Ref.	16.6 (9.6–23.6)	30.3 (11.5–49.1)
Whites (N = 6778)	4331	2162	285
CP mean values (mg L^{-1})	291.4	303.8	327.6
Difference (mg dL^{-1}), model 1	Ref.	11.7 (8.5–14.9)	30.4 (22.9–37.9)
Difference (mg dL^{-1}), model 2	Ref.	11.7 (8.5–14.9)	29.8 (22.5–37.2)
	GG	GT	TT
rs13072552			
African Americans (N = 1661)	550	824	287
CP mean values (mg L^{-1})	308.9	309.2	323.9
Difference (mg dL^{-1}), model 1	Ref.	0.9 (− 5.9 to 7.6)	13.6 (4.5–22.7)
Difference (mg dL^{-1}), model 2	Ref.	1.1 (− 5.7 to 7.8)	13.6 (4.6–22.6)
Whites (N = 6778)	5830	907	41
CP mean values (mg L^{-1})	293.6	314.3	340.3
Difference (mg dL^{-1}), model 1	Ref.	20.9 (16.5–25.2)	54.9 (35.8–74.0)
Difference (mg dL^{-1}), model 2	Ref.	21.1 (16.8–25.4)	53.8 (34.9–72.7)

Model 1: adjustment for age, sex, race, body mass index, hormone replacement therapy, and principal components of ancestry (in African Americans). Model 2: model 1 + adjustment for diabetes mellitus, systolic blood pressure, activated partial thromboplastin time, von Willebrand factor, D-dimer, factor VIII, factor XI, and high-sensitivity C-reactive protein.

causal role of CP in VTE risk, although statistical power to rule out a small effect was limited.

A previous publication from the Malmö Preventive Project in southern Sweden, including 6068 participants, explored whether raised levels of inflammation-sensitive plasma markers (fibrinogen, haptoglobin, CP, α_1-antitrypsin, and orosomucoid) were associated with increased VTE risk. They did not find any association between these biomarkers and VTE risk [11].

In contrast to this previous study, we found a positive association of CP concentration with VTE in a large, middle-aged, biracial cohort of men and women. After adjustment for several VTE risk factors and different biomarkers, higher circulating CP concentrations remained associated with increased VTE incidence.

Multiple clinical and molecular lines of evidence suggest a close link between inflammation, thrombosis activation, and VTE [18–22]. Inflammation increases the production of procoagulant factors, activating blood coagulation and inhibiting the fibrinolytic pathway [23]. During endothelial dysfunction, platelet-activating factor and endothelin-1 are released, promoting vasoconstriction, whereas production of FV, VWF, plasminogen activator inhibitor-1 and tissue factor augments thrombosis. Furthermore, endothelial cells increase the number of adhesion molecules in the surface, promoting the activation of leukocytes. This event initiates and amplifies inflammation and thrombosis [24].

It has been reported that inflammation, with the subsequent overproduction of reactive oxygen species (ROS), is associated with VTE risk [18–22]. CP has been suggested to have proinflammatory effects on vascular cells, both oxidative and antioxidative functions having been reported. Therefore, hypothetically, the overproduction of ROS and vascular inflammation could be a cause of VTE in the presence of higher CP concentrations.

In relation to C-reactive protein (CRP), we are uncertain why single measures of CRP were previously associated with incident VTE [9,25] but changes in these biomarkers were not, and this appears to be somewhat dependent on the follow-up time [26]. One possibility is that the original findings were spurious, because of some unrecognized confounding variable. The lack of longitudinal associations between CRP and VTE incidence suggest that this protein is not a risk factor for VTE. This is supported by the study of Zacho *et al.*, in which genetically elevated CRP levels were not associated with increased VTE risk [27].

During an inflammatory process, multiple factors are involved. Numerous inflammatory markers could be analyzed individually as potential VTE risk factors. Further studies would be needed to determine whether an elevated CP concentration is a causal risk factor or merely a risk marker for VTE.

Strengths and limitations

Strengths of the study include the large sample size and power to measure overall associations between CP concentration and VTE. However, there are a few limitations. Although the LITE study validated VTEs, some VTE cases treated in outpatient settings are missed [14]. In addition, there may be some misclassification of CP

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Table 4 Hazard ratios (95% confidence intervals) for the associations of the rs11708215 and rs13072552 single-nucleotide polymorphisms with venous thomboembolism (VTE) risk

	AA	AG	GG
rs11708215			
African Americans (N = 1661)	1225	393	43
VTE cases	61	25	2
Model 1	1 (ref.)	1.31 (0.82–2.10)	0.90 (0.22–3.72)
Model 2	1 (ref.)	1.28 (0.80–2.06)	1.06 (0.25–4.41)
Whites (N = 6778)	4331	2162	285
VTE cases	132	86	8
Model 1	1 (ref.)	1.31 (1.00–1.72)	0.89 (0.43–1.81)
Model 2	1 (ref.)	1.31 (1.00–1.72)	0.89 (0.43–1.82)
	GG	**GT**	**TT**
rs13072552			
African Americans (N = 1661)	550	824	287
VTE cases	21	50	17
Model 1	1 (ref.)	1.52 (0.91–2.53)	1.40 (0.73–2.69)
Model 2	1 (ref.)	1.54 (0.91–2.61)	1.57 (0.81–3.04)
		GT/TT	
Whites (N = 6778)	5830	948	
VTE cases	191	35	
Model 1	1 (ref.)	1.12 (0.78–1.61)	
Model 2	1 (ref.)	1.11 (0.77–1.59)	

Model 1: adjustment for age, sex, race, body mass index, hormone replacement therapy and principal components of ancestry (in African Americans). Model 2: model 1 + adjustment for diabetes mellitus, systolic blood pressure, activated partial thromboplastin time, von Willebrand factor, D-dimer, factor VIII, factor XI, and high-sensitivity C-reactive protein.

Table 5 Differences in ceruloplasmin (CP) concentration and venous thromboembolism (VTE) risk by number of CP-increasing alleles in rs11708215 and rs13072552

	No risk alelles	One risk alelle	Two risk alelles	Three to four risk alelles
N	4499	2497	1253	190
CP (mg L^{-1})	291.0	303.7	317.7	332.1
VTE cases	133	117	59	5
African Americans				
Difference in CP concentration (95% confidence intervals)				
N	353	791	466	51
VTE cases	10	46	29	3
Model 1	Ref.	11.3 (3.5–19.2)	23.4 (14.7–32.1)	32.5 (14.2–50.9)
Model 2	Ref.	11.8 (4.0–19.7)	23.8 (15.1–32.4)	32.9 (14.7–51.2)
Hazard ratios (95% confidence intervals)				
Model 1	1 (ref.)	2.03 (1.02–4.04)	2.11 (1.02–4.04)	1.73 (0.47–6.37)
Model 2	1 (ref.)	2.26 (1.11–4.60)	2.31 (1.10–4.87)	2.24 (0.60–8.38)
Whites				
Difference in CP concentration (95% confidence intervals)				
N	4146	1706	787	139
VTE cases	123	71	30	2
Model 1	Ref.	9.9 (6.5–13.5)	23.4 (18.7–28.11)	39.3 (28.8–49.8)
Model 2	Ref.	10.1 (6.6–13.5)	23.2 (18.6–27.9)	39.2 (28.8–49.5)
Hazard ratios (95% confidence intervals)				
Model 1	1 (ref.)	1.39 (1.04–1.86)	1.28 (0.86–1.91)	0.48 (0.12–1.94)
Model 2	1 (ref.)	1.38 (1.03–1.85)	1.29 (0.87–1.93)	0.45 (0.11–1.83)

Model 1: adjustment for age, sex, race, body mass index, hormone replacement therapy, and principal components of ancestry (in African Americans). Model 2: model 1 + adjustment for diabetes mellitus, systolic blood pressure, activated partial thromboplastin time, von Willebrand factor, D-dimer, factor VIII, factor XI, and high-sensitivity C-reactive protein.

concentrations, as there is no follow-up information on circulating CP concentrations after visit 4. As a result, if the CP measures changed over time, it would tend to bias our HRs (presumably towards 1). Power is poor for race-specific findings and for the SNP associations. We considered these SNPs because they have been previously

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Table 6 Differences in ceruloplasmin (CP) concentration, hazard ratios (HRs) and 95% confidence intervals (CIs) of venous thromboembolism (VTE) by haplotypes of rs11708215 and rs13072552

Haplotype (rs11708215/rs13072552)	*N*	VTE events	Ceruloplasmin (mg L^{-1})	Difference in CP (adjusted)*	HR (95% CI) of AF (adjusted)*
African Americans (n = 1661)					
AA/GG	353	10	298.4	Ref.	1 (Ref.)
AA/GT	621	36	305.3	7.6 (− 0.5 to 15.7)	2.19 (1.05–4.55)
AA/TT	251	15	323.4	22.7 (12.6–32.9)	2.35 (1.02–5.39)
AG/GG	170	10	324.8	26.9 (15.6–38.1)	2.50 (1.02–6.13)
AG/GT	188	13	321.8	22.4 (11.5–33.3)	2.36 (1.01–5.50)
AG/TT	35	2	326.9	30.5 (8.9–51.9)	2.01 (0.43–9.44)
GG/xx†	43	2	339.3	38.9 (19.4–58.4)	1.98 (0.43–9.26)
Whites (n = 6778)					
AA/GG	4146	123	290.4	Ref.	1 (Ref.)
AA/GT	183	9	311.0	23.6 (14.5–32.7)	1.49 (0.76–2.94)
AG/GG	1523	62	299.7	8.5 (4.8–12.1)	1.37 (1.01–1.86)
AG/GT	624	24	312.4	22.6 (17.4–27.7)	1.32 (0.85–2.04)
GG/GG	161	6	318.8	24.7 (15.1–34.4)	1.23 (0.54–2.79)
GG/GT	100	2	332.3	33.5 (21.3–45.6)	0.65 (0.16–2.62)
xx/TT‡	41	0	340.3	56.7 (37.9–75.6)	

*Adjusted for variables in model 1 +model 2 + principal components in African Americans. †GG/xx: 27 GG/GG (one VTE case), 15 GG/GT (one VTE case), and one GG/TT (no VTE cases). ‡xx/TT: two AA/TT (no VTE cases), 15 AG/TT (no VTE cases), and 24 GG/TT (no VTE cases).

associated with CP concentrations (rs13072552 in an ARIC genome-wide association study and rs11708215 in a candidate SNP analysis) [12]; there could be other variants related to CP concentrations that have not been identified yet. Finally, the hemostatic factors were not measured at visit 4, so there could be residual confounding. Finally, we could not take into account acute risk factors for VTE (immobility, surgery, etc.).

Conclusion

High circulating CP concentrations are associated with an increased VTE incidence. The two SNPs studied were associated with increased CP concentrations in both whites and African Americans. The presence of the CP-increasing alleles in rs11708215 and rs13072552 was not significantly associated with VTE risk in either race group. Our results suggest that CP may be one of many inflammatory intermediaries involved in the development of VTE, or at least may indicate a patient at risk for VTE.

Addendum

A. Alonso contributed significantly to the design of the study, reviewed the study proposal, and participated in drafting the manuscript and in each subsequent revision. A. Folsom reviewed the study proposal, participated in drafting the manuscript, and critically reviewed the manuscript. N. Roetker designed the database and reviewed the paper. F. Norby reviewed the paper and provided many recommendations and useful ideas. A. Arenas analyzed the database, interpreted the results obtained, and wrote and updated the manuscript following the suggested recommendations from other authors.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Hazard ratios (95% confidence intervals) for the association between CP concentration and VTE risk in women, ARIC, 1996–2011

Table S2. Hazard ratios (95% confidence intervals) for the association between CP concentration and VTE risk in men, ARIC, 1996–2011

Table S3. Hazard ratios (95% confidence intervals) for the association between CP concentration and VTE risk with the follow-up time limited to 11 years

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C. Ceruloplasmin and Coronary Heart Disease. A systematic review

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Review

Ceruloplasmin and Coronary Heart Disease-A Systematic Review

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Abstract: Several studies indicate that oxidative stress might play a central role in the initiation and maintenance of cardiovascular diseases. It remains unclear whether ceruloplasmin acts as a passive marker of inflammation or as a causal mediator. To better understand the impact of ceruloplasmin blood levels on the risk of cardiovascular disease, and paying special attention to coronary heart disease, we conducted a search on the two most commonly used electronic databases (Medline via PubMed and EMBASE) to analyze current assessment using observational studies in the general adult population. Each study was quality rated using criteria developed by the US Preventive Services Task Force. Most of 18 eligible studies reviewed support a direct relationship between ceruloplasmin elevated levels and incidence of coronary heart disease. Our results highlight the importance of promoting clinical trials that determine the functions of ceruloplasmin as a mediator in the development of coronary heart disease and evaluate whether the treatment of elevated ceruloplasmin levels has a role in the prognosis or prevention of this condition.

Keywords: ceruloplasmin; coronary heart disease; inflammation

1. Introduction

Cardiovascular disease (CVD) is the largest cause of death worldwide in developed countries. As a diagnostic category, CVD includes various areas: coronary heart disease (CHD), manifested by myocardial infarction (MI) or angina pectoris; cerebrovascular disease, manifested by stroke and transient ischemic attack; high blood pressure; peripheral artery disease and death by any of the above causes [1].

Despite the fact that the mortality from CHD has decreased over the last few decades in western countries, it still causes about one-third of all deaths in people over 35 years [2–4]. Although current guideline-guided CHD therapy has lowered both recurrence and death rates, people with CHD remain at high risk for these complications. One third of all CHD with known, controlled risk factors will have a recurrence in the following 10 years [5]. This is called residual risk, and many approaches have been taken to tackle it. One of the most important fields of research in this area is the search for additional biomarkers which may help to detect or be an early predictor of those cases which will develop a worse prognosis despite controlled risk factors.

Inflammation and oxidative stress are two of the processes involved in the development of atherosclerosis and CHD. Oxidative stress is believed to be a consequence of increased circulating neurohormones and hemodynamic disorder. Impairment of cardiac function could be caused by a redox balance disorder, an oxidative damage to cellular molecules, or a damage in cell signaling, compromising the cell survival and leading to death [6,7].

Ceruloplasmin (CP) belongs to the α2-glycoprotein fraction of plasma proteins. It is synthesized in the liver, incorporating copper, mainly from the diet, and accounts for 95% of the total circulating copper in healthy adults. Apart from playing a role in copper and iron metabolism, CP is an acute-phase reactant that may work as an antioxidant but can also generate free radicals that may lead to several illnesses [8,9]. It is interesting that most of the plasma copper that will end up building CP comes from dietary copper consumed weeks or months ago, not from recent meals, so it will take some time for CP to reflect changes involving copper availability in the diet.

Oxidative stress might play a central role in the initiation of CVD, but it remains unclear whether CP acts as a passive marker of inflammation or as a causal mediator in its development. Reviewing the scientific literature, it is clear that elucidating the effect of CP on CHD is a difficult task. For this reason, we carried out the first systematic review which provides evidence from the observational studies involving the effect of CP over the last three decades, in an attempt to better understand its impact on the risk of CHD.

2. Methods

We conducted a search of the two most commonly used electronic databases (Medline via PubMed and EMBASE) to analyze current assessment in observational studies. We identified the records using the following keywords: "Cardiovascular" OR "coronary" OR "heart" OR "angina" OR "myocardial" OR "infarction" AND "ceruloplasmin." Equivalent free-text terms were used. In this review, we were interested in exploring the evidence of CP and CHD after 1990.

The search resulted in 1407 records which were categorized and screened independently by AAL and LLP (differences resolved by JDL). When analyzing original articles, the authors decided whether the item was relevant or not, based on the title and the abstract. If considered pertinent, the referenced articles included in the item were added to the list of potential articles to include in this review. To assess the validity of each of these studies, we reviewed all the related articles and evaluated the quality of each study on the basis of criteria created by the third USPSTF (US Preventive Services Task Force) (Table 1) [10].

We removed all articles written in languages other than English, duplicated records, reviews, conference abstracts and letters, studies on a pediatric population, studies on animals and unrelated articles, resulting in 18 eligible studies (Table 2). This is illustrated as a flow-chart according to the PRISMA statement in Figure 1.

Table 1. US Preventive Services Task Force Quality Rating Criteria *.

Cohort studies
Criteria Initial assembly of comparable groups: cohort studies—consideration of potential confounders with either restriction or measurement for adjustment in the analysis; consideration of inception cohorts Maintenance of comparable groups (including attrition, crossovers, adherence, contamination) Major differential loss in follow-up or overall high loss in follow-up Measurements: equal, reliable and valid (including masking of outcome assessment) Clear definition of interventions Important outcomes considered Definition of ratings on the basis of above criteria **Good** Meets all criteria: comparable groups are assembled initially and maintained throughout the study (follow-up at least 80%); reliable and valid measurement instruments are used and applied equally to the groups; important outcomes are considered, and appropriate attention is given to confounders in analysis. **Fair** Any or all of the following problems occur, without the important limitations noted in the "poor" category: generally comparable groups are assembled initially but some question remains about whether some (albeit not major) differences occurred in follow-up; measurement instruments are acceptable (albeit not the best) and are generally applied equally; some but not all important outcomes are considered, and some but not all potential confounders are accounted for. **Poor** Any of the following major limitations exists: groups assembled initially are not close to being comparable or maintained throughout the study; unreliable or invalid measurement instruments are used, and key confounders are given little or no attention.
Case-control studies
Criteria Accurate ascertainment of cases Non-biased selection of cases/controls with exclusion criteria applied equally to both Response rate Diagnostic testing procedures applied equally to each group Measurement of exposure accurate and applied equally to each group Appropriate attention to potential confounding variables Definition of ratings on the basis of the above criteria **Good** Appropriate ascertainment of cases and non-biased selection of case and control participants; exclusion criteria applied equally to cases and controls; response rate equal to or greater than 80%; diagnostic procedures and measurements accurate and applied equally to cases and controls, and appropriate attention to confounding variables. **Fair** Recent, relevant, without major apparent selection or diagnostic work-up bias but with response rate less than 80% or attention to some but not all important confounding variables. **Poor** Major selection or diagnostic work-up biases, response rates less than 50%, or inattention to confounding variables.

* Adapted from Humphrey et al. [11].

Table 2. Summary of the articles included in this review.

Authors and Year of Publication	*Study Design, Population; Age (y; Mean ± Standard Desviation)*	*Sample Size, Cases/Controls*	*Follow-Up in Years (If Applicable)*	*Outcomes Evaluated*	*Main Findings Related to Ceruloplasmin*	*Quality of Study*	*Supports a Direct Relationship between Higher Ceruloplasmin (CP) Levels and Coronary Heart Disease (CHD) Risk (Yes/No)*
Reunanen et al. [12]; *1992*	Nested case-control, men and women; 59 (mean)	104/104	11.0	Incidence of MI and stroke	Higher serum CP levels are a risk factor for myocardial infarction (MI). Adjusted OR in the highest tertile: 3.1 (1.3–7.6 95% confidence interval (CI)	Good	Yes
M. Manttari et al. [13]; *1994*	Nested case-control, men; 49.3 ± 4.4 (cases); 47.2 ± 4.7 (controls)	136/136	5.0	Non-fatal myocardial infarction or cardiac death	There was an increase in coronary risk in patients with rising CP. The risk in the highest tertile was double (OR 2.1; 1.1–4.2 95% CI) that of the lowest. The risk of high CP was influenced by lipoprotein cholesterol concentrations, with an odds ratio of 2.4 (1.3–4.4 95% CI) in subjects with high low-density lipoprotein cholesterol and of 11.3 (2.5–52.2 95% CI) in subjects with low high-density lipoprotein cholesterol.	Good	Yes
Mori et al. [14]; *1995*	Cohort, men and women, 57.8 ± 9.7; 61.2 ± 9.3 respectively	225	4.1	Severity of coronary atherosclerosis in patients undergoing coronary angiography. (Gensini Score)	CP can be an independent risk factor for coronary atherosclerosis and determine the severity of the disease.	Fair	Yes
Enbergs et al. [15]; *1998*	Cohort, men and women, 55.1 ± 9.6; 54.6 ± 10.0 respectively.	275	1.0	The extent of CHD assessed by three scores (Vessel score, stenosis score and extent score)	Serum CP levels were not confirmed as risk factor for the extent of CHD.	Fair	No
Klipstein-Grobusch et al. [16]; *1999*	Nested case-control, men and women; 76.4 ± 8.7 (cases); 76.8 ± 9.0 (controls)	83/127	4.0	Incidence of MI	Risk of MI for the highest compared with the lowest quartile of CP was 2.46 (1.04–6.00 95% CI). After adjustment for C-reactive protein and leucocyte count, the excess risk was reduced by 33% suggesting that the association between serum CP and CHD may be attributed to inflammation processes.	Good	Yes

Table 2. *Cont.*

Authors and Year of Publication	*Study Design, Population; Age (y; Mean ± Standard Desviation)*	*Sample Size, Cases/Controls*	*Follow-Up in Years (If Applicable)*	*Outcomes Evaluated*	*Main Findings Related to Ceruloplasmin*	*Quality of Study*	*Supports a Direct Relationship between Higher Ceruloplasmin (CP) Levels and Coronary Heart Disease (CHD) Risk (Yes/No)*
G. Engström et al. [17]; *2003*	Cohort, men; mean approximately 46.9	6075	18.1 ± 4.3 years	Incidence of MI	CP levels increased the Incidence of MI. The relative risk in the highest quartile of low-risk group were 1.00 (reference), 1.9 (95% CI 0.8–4.2), 1.8 (95% CI 0.6–5.4), and 2.9 (95% CI 1.05–8.1), respectively, for men with an increasing number of inflammation-sensitive plasma proteins (ISPs) (0, 1, 2 and ≥ 3 ISPs). On the other hand, in the high-risk group, relative risks (RRs) were 1.00, 1.4 (95% CI 0.9–2.2), 1.9 (95% CI 1.2–3.1), and 2.0 (95% CI 1.3–3.1), respectively, for men with an increasing number of ISPs (0, 1, 2 and ≥ 3 ISPs)	Good	Yes
G. Engström et al. [18]; *2004*	Cohort, men; 46.8 ± 3.7.	6075	18.7 ± 4.2	Incidences of cardiovascular events (myocardial infarction, stroke, cardiovascular deaths), cardiac events (fatal or nonfatal myocardial infarction), and stroke	The age-adjusted relative risks in obese men were 2.1 (95% CI 1.4–3.4), 2.4 (95% CI 1.5–3.7), 3.7 (95% CI 2.3–6.0), and 4.5 (95% CI 3.0–6.6), respectively for men with an increasing number of ISPs (0, 1, 2 and ≥ 3 ISPs)	Good	Yes
G. Engström et al. [19]; *2004*	Cohort, men; 46.8 ± 3.7.	6075	19	Nonfatal MI or death from CHD	A higher number of CHD deaths was noted in men who had presented a low-grade inflammation during many years before. Of the 680 men with a coronary event, 197 died the first day and 228 died within 28 days. The proportions who died the first day were 26%, 25%, 29%, and 35%, respectively, for men with an increasing number of ISPs (0, 1, 2 and ≥ 3 ISPs). The corresponding proportions who died within 28 days were 30%, 31%, 34%, and 38%, respectively	Good	Yes

Table 2. *Cont.*

Authors and Year of Publication	*Study Design, Population; Age (y; Mean ± Standard Desviation)*	*Sample Size, Cases/Controls*	*Follow-Up in Years (If Applicable)*	*Outcomes Evaluated*	*Main Findings Related to Ceruloplasmin*	*Quality of Study*	*Supports a Direct Relationship between Higher Ceruloplasmin (CP) Levels and Coronary Heart Disease (CHD) Risk (Yes/No)*
Verma et al. [20]; *2005*	Cohort, men and women; 50–59	250		Severity of coronary artery disease (CAD) and modifiable CAD risk factors	Verma et al. explored how serum levels of three antioxidants (vitamin C, bilirubin and CP) were related to CHD risk factors. A 7–18% decrease was observed in CHD patients with severe disease with increasing serum levels of the three antioxidants. In the same line, a decrease of 14–20% was objectified in triple vessel disease and of 39% in MI occurred with increasing serum CP in CHD patients, compared to the non-MI group. An inverse relationship was found between the three antioxidants studied and coronary risk factor suggesting that greater care in traditional risk factors would maintain a high level of these antioxidants	Poor	No
Brunetti et al. [21]; *2008*	Cohort, men and women; 65.8 ± 11.25	123		Left ventricular systolic function during the early phase of acute MI	Systolic dysfunction in ST elevation acute MI patients seems to be associated with an inflammatory response featured by a rise in plasmatic concentration of acute-phase proteins (APPs); increase in APPs concentrations seems to own a short-term prognostic relevance. CP values were the most significant markers of acute heart failure when compared with patients without systolic dysfunction (40.1 ± 9.7 vs. 31.4 ± 7.6 mg/dL, $p < 0.001$).	Fair	Yes
Göçmen et al. [22]; *2008*	Case-control, men and women; 56.31 ± 2.74 (men); 54.23 ± 1.55 (women)	26/26		CAD	High CP and low albumin levels were found to be independent risk factors for CAD.	Poor	Yes
Kaur et al. [23]; *2008*	Case-control, men and women; 41–60	50/30		CAD	Increase in the levels of CP in patients of CAD (Mean ±SD, 48.93 ± 4.44 mg/dl as compared to controls (32.25 ± 4.67 mg %). CP could be a risk factor of CAD by modifying of Low-density lipoprotein (LDL) to an atherogenic form.	Poor	Yes

Table 2. *Cont.*

Authors and Year of Publication	*Study Design, Population; Age (y; Mean ± Standard Desviation)*	*Sample Size, Cases/Controls*	*Follow-Up in Years (If Applicable)*	*Outcomes Evaluated*	*Main Findings Related to Ceruloplasmin*	*Quality of Study*	*Supports a Direct Relationship between Higher Ceruloplasmin (CP) Levels and Coronary Heart Disease (CHD) Risk (Yes/No)*
Deepa et al. [24]; *2009*	Case-control, men; 43 (mean approximately)	100/50		Acute MI with Diabetes Mellitus (DM) and non-DM	CP levels were significantly higher in diabetic and non-diabetic MI patients as compared with controls ($p < 0.001$) suggesting that CP may act as an oxidative stress indicator.	Poor	Yes
Kumar et al. [25]; *2009*	Case-control, men and women; 61.8 ± 3.8 (cases); 60.5 ± 3.4 (controls)	165/165		MI	CP levels were higher in MI patients than controls.	Fair	Yes
Tang et al. [26]; *2010*	Cohort, men and women; 63± 11 approximately	3828	3.0	Subclinical myocardial necrosis	The presence of subclinical myocardial necrosis was associated with elevations in CP levels.	Good	Yes
Tang et al. [27]; *2012*	Cohort, men and women; 63± 11 approximately	4177	3.0	Incident major adverse cardiovascular events (MACE = death, MI, stroke) in stable cardiac patients.	Serum CP level was associated with higher risk of MI with a HR of 2.35, (95% CI 1.79–3.09) comparing the top quartile versus the lowest. CP remained independently predictive of MACE (HR 1.55, 95% CI 1.10–2.17). Genetic variants at the CP locus were not associated with prevalent or incident risk of CAD.	Good	Yes
T. B. Grammer et al. [28]; *2014*	Cohort, men and women; 62.5 ± 10 approximately	3253	4.0	Angiographic CAD and mortality from all causes and cardiovascular causes.	When the highest quartile for CP levels was compared to the lowest, HR for death from any cause was 2.63 (95% CI, 2.17–3.20), and HR for death from cardiovascular causes was 3.02 (95% CI, 2.36–3.86). The concentration of CP was therefore independently associated with increased risk of death from all and cardiovascular	Good	Yes
Xue Bao et al. [29]; *2018*	Cohort, men and women; mean 57 approximately	Sub-cohort 4658	17.7 ± 5.46	DM and CVD	CP levels, alpha1-antitrypsin and soluble urokinase plasminogen activator receptor predicted increased risk of CVD but not DM.	Good	Yes

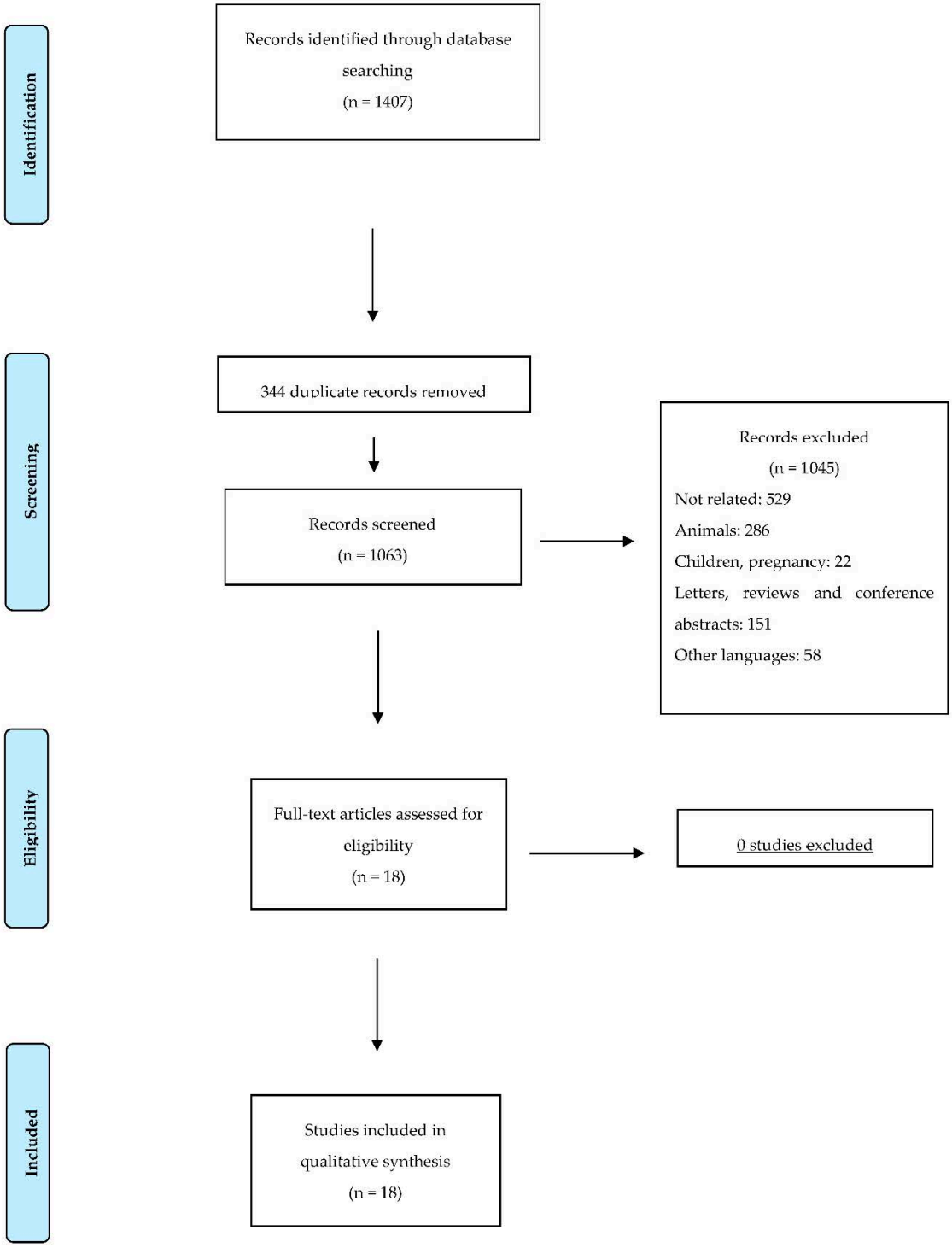

Figure 1. Flow diagram illustrating the search strategy used according to PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) statement.

3. Results

The probable relationship between the CP concentration in serum and the incidence of atherosclerosis and other cardiovascular conditions was first suggested in the 1950s [30]. High serum CP levels have been found in patients with arteriosclerosis [31], unstable angina [32], stroke and MI (15). Prospective studies have showed that serum CP may be an independent risk factor for cardiovascular

disease. A nested, case-control study by Reunanen et al. was the first to show that serum CP was positively associated with CHD [12]. In this study, which included 208 patients, the association between serum CP level and the subsequent incidence of MI was explored and higher serum CP concentration was found to be directly related with MI with an adjusted Odds Ratio (OR) of 3.1 (95% confidence interval (CI) 1.3–7.6) in the highest tertile compared to the lowest tertile. The same result was obtained by Mänttäri et al. in middle-aged patients with dyslipidemia, where there was a continuous, graded increase in coronary risk in patients with increasing CP. The risk in the highest tertile was double (OR 2.1; 95% CI 1.1–4.2) that of the lowest tertile, with an odds ratio of 2.4 (95% CI 1.3–4.4) in subjects with high low-density lipoprotein cholesterol and of 11.3 (95%CI 2.5–52.2) in subjects with low high-density lipoprotein cholesterol [13].

Because inflammation is recognized as a key player in atherosclerotic progression, Mori et al. separated the risk contributed by CP from that of inflammation (α1-antitrypsin, α1-acid glycoprotein, α2-macroglobulin, haptoglobin, fibrinogen, C4b binding protein, lipoprotein (a) and C-reactive protein (CRP)) and suggested that CP could serve as independent risk factor for coronary atherosclerosis and as a marker for the severity of disease [14]. In these terms, Klipstein-Grobusch et al. confirmed the association between serum CP levels and subsequent MI in a four-year follow-up study. The risk of MI for the highest compared with the lowest quartile of CP was 2.46 (95% CI 1.04–6.00), a relationship that continued after making adjustments for other potential contributors, like C-reactive protein or leucocyte count (lowering a third of the effect), thus supporting the theory that the excess CP itself contributes substantially to the risk of CHD [16].

Five inflammation-sensitive plasma proteins (ISPs; fibrinogen, oroso-mucoid, α1-antitrypsin, haptoglobin, and CP) were measured in 6075 healthy patients from the Malmö Study with a mean follow-up of 18 years. Engstrom et al. carried out several studies in this population to determine the association of ISPs with incidence of CHD, the cardiovascular risk in overweight or obese men and the fatality of future coronary events.

In the first study, the incidence of MI was related to ISPs. Patients were categorized into low-risk and high-risk groups according to traditional risk factors. The relative risk in the highest quartile of low-risk group were 1.00 (reference), 1.9 (95% CI 0.8–4.2), 1.8 (95% CI 0.6–5.4), and 2.9 (95% CI 1.05–8.1), respectively, for men with an increasing number of ISPs (0, 1, 2 and ≥3 ISPs). On the other hand, in the high-risk group, RRs were 1.00, 1.4 (95% CI 0.9–2.2), 1.9 (95% CI 1.2–3.1), and 2.0 (95% CI 1.3–3.1), respectively, for men with an increasing number of ISPs (0, 1, 2 and ≥3 ISPs) [17].

The idea that ISPs may modify the cardiovascular risk in overweight or obese men was explored in a later study. High ISPs levels were associated with an increased cardiovascular risk. The age-adjusted relative risks in obese men were 2.1 (95% CI 1.4–3.4), 2.4 (95% CI 1.5–3.7), 3.7 (95% CI 2.3–6.0), and 4.5 (95% CI 3.0–6.6), respectively, for men with an increasing number of ISPs (0, 1, 2 and ≥3 ISPs) [18].

A third study was performed to determine whether low-grade inflammation (measured by ISP levels) in healthy men predicted the fatality of future coronary events. A higher number of CHD deaths was noted in men who had presented a low-grade inflammation during many previous years. Of the 680 men with a coronary event, 197 died on the first day and 228 died within 28 days. The proportions who died on the first day were 26%, 25%, 29%, and 35%, respectively, for men with an increasing number of ISPs (0, 1, 2 and ≥3 ISPs). The corresponding proportions who died within 28 days were 30%, 31%, 34%, and 38%, respectively. Although we cannot infer the exact contribution of CP to these studies, it seems that it may interact with some of the other inflammatory markers, and might exert its hypothetical effect by means of inflammation [19].

Systolic dysfunction in acute ST-elevation MI patients seems to be associated with an inflammatory response characterized by a rise in plasma concentration of ISPs (α1antitrypsin, α1glycoprotein, haptoglobin, CP and C-reactive protein) and appears to provide a short-term prognostic relevance. This was the conclusion reached by Brunetti et al., where the incidence of CHD correlated with the ISP values, and CP values were the most significant markers of acute heart failure when compared with patients without systolic dysfunction (40.1 ± 9.7 vs. 31.4 ± 7.6 mg/dL, $p < 0.001$) [21].

Göçmen et al. found increased CP levels in CHD patients. An increase in oxidants could be a possible cause of this increase in CP in CHD patients. In their study, serum CP levels were reported to be an independent risk factor for cardiovascular diseases [22]. There was a consistent increase in the levels of CP in CHD patients (48.93 ± 4.44 mg %) compared to controls (32.25 ± 4.67 mg %) in a study by Kaur et al., where statistical analysis revealed significantly increased CP levels in all the subgroups (acute MI, unstable angina and stable angina) [23].

CP levels were elevated in patients with acute MI and Diabetes Mellitus (DM) compared to non-diabetics with MI, possibly because of the greater degree of inflammation in these patients. However, regardless of this factor, CHD patients showed more inflammation, and consequently higher CP levels, than controls [24].

In a prospective case-control study by Kumar et al., designed to identify and evaluate potential risk factors in normolipidemic patients with an acute MI, CP was again found to be higher in cases than in the controls, in a study that also evaluated other potential markers, such as lipoprotein a, CRP and fibrinogen [25].

Subclinical myocardial necrosis as a prognostic feature of long-term adverse cardiac events risk has been studied on several occasions. Tang et al. first explored it in 3828 patients undergoing elective diagnostic coronary angiography with troponin I levels below the cut-off for defining MI. Here, the authors studied the underlying mechanisms contributing to myocardial necrosis and the risk of major adverse cardiovascular events. CRP and CP were associated with subclinical myocardial necrosis after 3-years of follow-up [26]. Afterwards, Tang et al. studied the relationship between CP levels and the incidence of major adverse cardiovascular events (MACE = death, MI and stroke) in 4177 patients undergoing elective coronary angiography after 3-years of follow-up. They performed a genome-wide association study of CP to determine genetic variants that could be related to prevalent and incident cardiovascular risk. Serum CP level was associated with higher risk of MI with a HR of 2.35, (95% CI 1.79–3.09) when comparing the top quartile versus the lowest. CP remained independently predictive of MACE (HR 1.55, 95% CI 1.10–2.17). Genetic variants at the CP locus were not associated with prevalent or incident risk of CAD [27].

Similar conclusions were reached by Grammer et al., who examined whether serum copper and CP concentrations were associated with angiographic CAD and mortality from all causes and cardiovascular causes. When the highest quartile for CP levels was compared to the lowest, HR for death from any cause was 2.63 (95% CI, 2.17–3.20), and HR for death from cardiovascular causes was 3.02 (95% CI, 2.36–3.86). The concentration of CP was therefore independently associated with increased risk of death from all and cardiovascular causes [28].

Finally, in a sub-cohort of 4658 participants in the Malmö Diet and Cancer study, seven inflammatory markers were studied to evaluate their incidence in DM and CVD (coronary events, including fatal and nonfatal MI or stroke). CP, among other molecules, predicted an increased risk of CVD but not of DM [29].

However, not all the studies evaluating the relationship between serum CP and CHD have produced the same results. For example, Enbergs et al. found no such relation when attempting to relate CP with the extent of atherosclerosis in coronarography. However, there were a number of limitations as regards patient selection in this investigation [15].

Along similar lines, Verma et al. explored how serum levels of three antioxidants (vitamin C, bilirubin and CP) were related to CHD risk factors. A 7–18% decrease was observed in CHD patients with severe disease, with increasing serum levels of the three antioxidants. Similarly, a decrease of 14–20% was objectified in triple vessel disease and of 39% in MI with increasing serum CP in CHD patients, compared to the non-MI group. An inverse relationship was found between the three antioxidants studied and coronary risk factor suggesting that greater care in traditional risk factors would maintain a high level of these antioxidants [20].

4. Discussion

Our systematic review shows an association between elevated CP levels and CHD that is unrelated to Framingham risk factors. As mentioned above, traditional risk factors are thought to account for most CHDs, although 15% to 20% of patients have no identified risk factors and this entails the impossibility of an adequate treatment to prevent a first event. For this reason, the scientific community has tried to identify other modifiable risk factors which help to predict an important number of CHD events. For this, CP, a protein closely linked to inorganic nutrient copper, is a strong, biologically-plausible candidate.

Most of the studies analyzed in the present work showed a uniformity in their results, with the exception of the study by Verma et al., towards a direct relationship between serum CP levels and incidence of CHD or cardiovascular events. The higher serum CP level the patient has, the more likely the patient is to suffer some complications.

The mechanism by which CP may influence cardiovascular disease is still unknown. It seems that reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, may be important in the main underlying mechanisms. It is hypothesized that, with high ROS levels, antioxidant systems such as superoxide dismutase, catalase and glutathione are overwhelmed and the structural integrity of CP is damaged [33].

CP is involved in iron removal from the cells and its dysfunction, in particular a loss of its ferroxidase activity, may lead to an accumulation of iron in tissues. Moreover, this loss of function produces unbound or free copper, which, together with the iron, can produce pathogenic effects in the cell such as apoptosis, cell toxicity, cell replication, greater oxidative stress and pathogenic gene activation [9]. It has been shown in a murine model that copper from myocardium is also released to the blood during the ischemic process thanks to the increase of copper metabolism MURR domain 1 (COMMD1), a Cu transport chaperone [34]. Therefore, the increase of copper in blood, and secondarily of CP caused by MI, seems not completely associated with an inflammatory response.

Oxidation of Low-density lipoproteins (LDLs) leads to the initiation or acceleration of the atherosclerotic process inducing the formation of autoantibodies against oxidized LDL (anti-oxLDL). The presence of CP and other acute-phase proteins in atherosclerotic lesions seems to incriminate a pathway involving lipid and lipoprotein oxidation, which plays an important role in the etiology of CHD. In fact, several study groups have provided evidence that CP is a potent catalyst of LDL oxidation in vitro and in vivo [35–37]. The study published by Awadallah et al. deserves particular consideration being the only study demonstrating an association between the concentrations of anti-oxLDL and those of CP and copper in patients with CVD [38]. Serum concentrations of CP and LDL lipid peroxides were correlated with atherosclerotic process and restenosis in patients undergoing endarterectomy [39]. Therefore, these studies suggest that CP may play a role in the oxidation of LDL in vivo.

To summarize and tie all the above threads together, CP may promote an inflammatory environment, with the associated defense mechanisms activating the ROS cascade by directly or indirectly producing the oxidation of LDL.

Another important mechanism by which CP may exert an effect in atherosclerosis is by affecting the nitric oxide (NO) pathway, which plays a key role in normal cardiac physiology and a protective role in the ischemic and failing heart [40,41]. CP can exert an important prooxidant function, related to NO oxidase, which may decrease NO bioavailability in plasma through its catalytic activity under given conditions. This has been shown in several studies after CP immuno-depletion and in humans with aceruloplasminemia with no NO oxidase activity [42,43].

Although most of the studies support a direct association between serum CP and CHD, some studies have questioned this association. In this context, CP was shown by Chapman et al. to have another antioxidant property through inhibition of myeloperoxidase, which stops free radical production [44], and Verma et al. established an inverse relationship between serum CP levels and coronary risk factors [20]. However, this study also had several limitations: CP was measured with an old technique and the international system of units was not followed; the baseline characteristics of the

patients were not defined and only cholesterol and triglycerides were compared as CHD risk factors with the three antioxidants. Finally, Enbergs et al. did not find any quantitative relationship between CP and the coronary atherosclerosis observed through angiography. Nevertheless, the paper also had one very critical limitation, in that it excluded patients with inflammatory conditions, thus consequently ruling out a clinical population with increased CP levels.

5. Limitations

As in any review, publication bias may have shifted the review towards positive findings, due to the fact that positive results have more chances of being published. Nevertheless, the fact that a good proportion of our results come from studies where CP is not the main objective, and results of those article are aligned with the articles in which CP is the main focus, supports our results. Another limitation is that we were not able to establish a specific cut-off point of CP to indicate a higher CHD risk. This is because studies are not homogeneous and so neither are their results. The limits established in our search strategy may also have limited our results. Although some articles written in other languages may be valid, limiting the articles included to those written in English is a common feature in Reviews. Finally, we reviewed here the articles published in the last 30 years, but not earlier studies.

6. Conclusions

Most of the studies reviewed in this article support a direct relationship between elevated CP levels and CVD. Patients with high CP serum levels were more likely to have a CV event, especially a CHD. However, CP cannot currently be considered as a coronary risk factor that may provide any value in prioritizing preventive interventions amongst those with unrecognized CVD or offer recommendations for people in secondary CHD prevention.

In view of the results of the observational studies included in this review, we believe that there is a basis supporting the importance of evaluating whether the treatment of elevated CP levels has a role in the prognosis or prevention of CHD.

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Chapter 5

RESULTS AND DISCUSSION

CHAPTER 5: RESULTS AND DISCUSSION

A. Relationship of plasma ceruloplasmin and the incidence of atrial fibrillation

Our first publication (Chapter 4.A) is the largest prospective study to date showing that higher concentrations of circulating CP are associated with AF. Moreover, in this study we found that genetic variants (SNPs rs11708215 and rs13072552) in or near the CP gene in chromosome 3, also influence circulating concentrations of CP. These SNPs, associated with higher CP concentrations, were associated with lower risk of AF in whites.

A prior study from the Malmö Preventive Project in southern Sweden, which included 3900 individuals, found a link between CP levels and the occurrence of AF (20). The findings of our investigation confirm those previous results and extend them to a large, middle-aged, multiracial cohort of men and women. Higher levels of circulating CP were linked to occurrence AF after controlling for conventional risk variables and biomarkers.

When looking for possible mechanism underlying our findings, it has been suggested that a normal redox balance is essential for the correct cardiac rhythm homeostasis, and that CP may influence that redox balance. In fact, CP has been reported to possess both oxidative and antioxidative functions, depending on its structure (33). It has been seen that the overproduction of reactive oxygen species (ROS) produces a damage in CP structure making it dysfunctional (77). The dysfunction of CP in men drives to an accumulation of iron in tissues which is associated with AF (20).

Apart from deleterious iron effect, free copper can produce similar effects to that of free iron (apoptosis, pathogenic gene activation or cell toxicity among others).
In the context of inflammation, CP may activate the NO oxidase and catalytically consume NO, lowering its bioavailability in plasma. NO and NO synthases have been demonstrated to play a crucial function in normal cardiac physiology in a variety of animal and human investigations (78-80). NO is a cardioprotective agent because it inhibits oxidative stress, among other things. As a result, excessive amounts of CP in the

body can promote NO oxidase activity, resulting in a reduction in NO and shifting the balance towards an oxidative function. This oxidative stress may affect heart electrical activity, causing ion channel damage and eventually contributing to the onset of AF (81, 82).

Despite all the above, more studies are necessaries to support a direct causal role of CP on AF risk. Our statistical power was possibly limited.

B. Relationship of plasma ceruloplasmin and the incidence of venous thromboembolism

Our second published article (Chapter 4.B) studied the relationship of CP with VTE risk, being the largest study to date showing that a higher concentration of circulating CP is associated with increased risk for VTE. We found that the same SNPs, rs11708215 and rs13072552, were associated with circulating CP concentrations in both whites and African Americans.

Higher levels of inflammation-sensitive plasma markers (fibrinogen, haptoglobin, CP, 1-antitrypsin, and orosomucoid) were linked with increased VTE risk in a prior study from the Malmö Preventive Project in southern Sweden, which included 6068 individuals. They discovered no association between these biomarkers and the risk of VTE (71).

In contrast to the previous investigation, we discovered a relation between CP concentration and VTE in a large, multiracial, middle-aged cohort of men and women. After accounting for a variety of VTE risk variables and biomarkers, greater circulating CP concentrations were still associated to an elevated risk of VTE.

Even though VTE is not classified as a chronic systemic inflammatory illness like atherosclerosis; any inflammatory reaction, regardless of its source, can cause hypercoagulability and raise the risk of VTE. As we discussed in Chapter 2.B, multiple clinical and molecular lines of evidence show a tight connection between inflammation, thrombosis activation and VTE. (38, 83-86). Inflammation activates blood coagulation and inhibits the fibrinolytic pathway by increasing the production of procoagulant substances (87). Platelet-activating factor and endothelin-1 are produced during endothelial failure, causing vasoconstriction, whereas Factor V, Von Willebrand factor,

plasminogen activator inhibitor-1, and tissue factor increase thrombosis. Endothelial cells also increase the amount of adhesion molecules on the surface, which promotes leukocyte activation. Inflammation and thrombosis are triggered and amplified by this occurrence (65).

As a result, inflammation and the resultant overproduction of reactive oxygen species (ROS) are linked to the risk of VTE. Both oxidative and antioxidative activities have been found for CP, suggesting that it has proinflammatory effects on vascular cells. Therefore, in the presence of increased CP concentrations, overproduction of ROS and vascular inflammation might be a cause of VTE.

As we mentioned in the previous section (Chapter 5.A), we could not find evidence for a direct causative effect of CP in VTE risk because statistical power was limited.

C. Relationship of plasma ceruloplasmin and the incidence and prevalence of coronary heart disease

Our third article (Chapter 4.C) investigated the evidence supporting an association between elevated CP levels and CHD.

As mentioned in Chapter 1, the rationale for this approach was based on the residual risk, or, in other words, that although traditional risk factors are thought to account for most CHDs, 15% to 20% of patients suffer a CHD event (first or recurrent events) even though they have not identified risk factors. This is a critical situation, because it implies that, in this population there is an impossibility of an adequate primary or secondary prevention. For this reason, the scientific community aims to identify other modifiable risk factors which help to predict an important number of CHD events.

Most of the studies that we analyzed in our systematic review showed a uniformity in their results towards a direct relationship between serum CP levels and incidence of CHD or cardiovascular events. Patients who had higher serum CP levels are more likely to suffer cardiovascular complications.

Although the exact mechanisms by which CP may influence cardiovascular disease is still unknown, the available current data suggest that redox, inflammation and hemostatic systems can be responsible (33).

The presence of CP in atherosclerotic lesions appears to implicate a lipid and lipoprotein oxidation pathway, which is crucial in the pathogenesis of CHD. In fact, numerous investigations have shown that CP is a powerful LDL oxidation catalyst in vitro and in vivo (88-90). Oxidation of LDL leads to the initiation or acceleration of the atherosclerotic process, resulting in the production of autoantibodies against oxidized LDL (anti-oxLDL). The work by Awadallah et al. merits special attention since it is the first to show a correlation between anti-oxLDL and CP concentrations and copper blood concentrations in patients with CVD (91). According to this, blood concentrations of CP and LDL lipid peroxides are associated to the degree of atherosclerosis and restenosis in individuals following endarterectomy (92).

Apart from cardiac electrical activity alterations (Chapter 5.A), CP may have a role in atherosclerosis via influencing NO, which plays a crucial function in normal cardiac physiology as well as a protective role in ischemic and failing heart (79) Under some situations, CP can have a significant prooxidant effect, similar to NO oxidase, which can reduce NO bioavailability in plasma through its catalytic activity. This has been demonstrated in a number of experiments after CP immunodepletion and in individuals with aceruloplasminemia who do not have NO oxidase activity (78, 93).

Most of the studies reviewed (Chapter 4.C) supported a direct relationship between elevated CP levels and CVD. Patients with high CP serum levels were more likely to have a CV event, especially a CHD.

Chapter 6

CONCLUSIONS

CHAPTER 6: CONCLUSIONS

1) High levels of plasma ceruloplasmin are associated with increased incidence of atrial fibrillation.

2) High levels of plasma ceruloplasmin are associated with increased incidence of venous thromboembolism.

3) A systematic review of the current scientific evidence supports that high levels of plasma ceruloplasmin are associated to a higher risk of coronary heart disease.

Chapter 7

CURRICULUM VITAE

CHAPTER 7: CURRICULUM VITAE

The author of this thesis, Antonio Pablo Arenas de Larriva, was born on 25 of April in 1987 in Córdoba, Spain. He started medical school at the University of Cordoba in 2005, obtaining his medical degree in 2011. He completed his specialty training in Internal Medicine at the Reina Sofia University Hospital (Córdoba) in 2017. As internal medicine resident he started the research described in this thesis at the department of Internal Medicine, Lipids and Atherosclerosis Unit at the Reina Sofia University Hospital in Cordoba under the supervision of Prof. Jose López Miranda and Dr. Javier Delgado Lista. Before he obtained his title as Internal Medicine specialist, he spent six months at the University of Minnesota, (Minneapolis, United States of America) in 2016 under the supervision of Prof. A. Alonso Gutiérrez, where he collaborated investigating the role of ceruloplasmin in cardiovascular disease. In 2017, the author was included as researcher at the 'Instituto Maimónides de Investigación Biomédica de Córdoba' (IMIBIC) and obtained a Rio Hortega contract (Instituto de Salud Carlos III) in 2020 with a potential project in Big Data.

Master degree in Nutrition and Metabolism at the University of Cordoba in 2014. Currently, he is a collaborating researcher in an European project on Diabetes: POWER2DM and in a Spanish project related to fatty liver and its regression called PROMETEO.

Chapter 8

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