PUBLICATIONS OF
THE UNIVERSITY OF EASTERN FINLAND

Dissertations in Health Sciences

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Publications of the University of Eastern Finland
Dissertations in Health Sciences
No 534

Institute of Public Health and Clinical Nutrition, School of Medicine, Faculty of Health Sciences
University of Eastern Finland
Kuopio
2019

Distributor:
University of Eastern Finland
Kuopio Campus Library
P.O.Box 1627
FI-70211 Kuopio, Finland
www.uef.fi/kirjasto

Grano, 2019

ISBN: 978-952-61-3207-5 (print/nid.)
ISBN: 978-952-61-3208-2 (PDF)
ISSNL: 1798-5706
ISSN: 1798-5706
ISSN: 1798-5714 (PDF)

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Hämäläinen, Päivi
Markers of iron metabolism and adipose tissue dysfunction in metabolic syndrome and in association with lipoprotein particle size and concentration
Kuopio: University of Eastern Finland
Publications of the University of Eastern Finland
Dissertations in Health Sciences 534. 2019, 75 p.
ISBN: 978-952-61-3207-5 (print)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-3208-2 (PDF)
ISSN: 1798-5714 (PDF)

ABSTRACT

The components of metabolic syndrome (MetS), abdominal obesity, increased blood pressure, glucose intolerance and dyslipidemia, constitute a clustering of risk factors for type 2 diabetes and cardiovascular disease. The prevalence of MetS is increasing with the aging of the population and the prevalence of obesity. More information is needed on the factors affecting the progression of MetS and its transition to cardiovascular disease as well as on the markers that could help recognize the subjects at high risk in clinical work.

The purpose of this study was to investigate whether the markers of hypoxia and adipose tissue dysfunction as well as iron metabolism are associated with MetS and its components. Another aim was to test whether an atherogenic lipoprotein particle profile is associated with hemoglobin level. The selected markers for adipose tissue dysfunction were erythropoietin (EPO), hemoglobin and haptoglobin whereas ferritin and transferrin receptor (TFR) were tested for markers for iron metabolism in a cross-sectional study design. The association between changes in serum ferritin level and the development or resolution of MetS and its components were investigated during a 6.5-year follow-up period. Associations between nuclear magnetic resonance (NMR)-measured lipoprotein particles concentration and sizes and hemoglobin level were tested cross-sectionally.

The study population consisted initially of 1,294 inhabitants from the town of Pieksämäki, from five age cohorts (mean age 52 years) born in 1942, 1947, 1952, or 1962. The subjects were recruited from population data records and invited for a health care visit in 1997-1998 (baseline) and again in 2003-2004 (follow-up).

Higher hemoglobin, erythropoietin, haptoglobin and ferritin concentrations were associated with metabolic syndrome. Higher hemoglobin levels were related to all components of MetS whereas erythropoietin levels were related only with abdominal obesity. Higher ferritin levels were associated with triglycerides, abdominal obesity, elevated glucose or low high-density cholesterol. An increase in serum ferritin over the 6.5-year period was associated with development of MetS in both men and women. A change in ferritin level during the 6.5-year follow-up was associated with resolving or developing hyperglycemia, hypertriglyceridemia and the abdominal obesity components of MetS. Higher hemoglobin levels were associated with larger VLDL, smaller LDL, and smaller HDL particle sizes and increasing amounts of larger VLDL and smaller LDL particles.

In conclusion, the findings of this study suggest that erythropoietin concentrations can act as a marker for the adipose tissue dysfunction associated with MetS. Hemoglobin is a readily available laboratory parameter that could complement the risk assessment of patients with metabolic risk factors, possibly suggesting a higher CVD risk profile. Ferritin levels can act as a marker in the detection of the MetS as well as in the follow-up of patients with MetS or its components. The limitations concerning the use of ferritin are other conditions influencing its levels and should be taken into account.

National Library of Medicine Classification: QU 85.6, WD 200.5.I7, WD 210, WH 150, WH 190, WK 810, WK 880
Medical Subject Headings: Metabolic syndrome; Ferritins; Hemoglobins; Erythropoietin; Lipoproteins; Iron/metabolism; Diabetes Mellitus, Type 2; Dyslipidemias; Obesity, Abdominal; Receptors, Transferrin; Risk Factors

Hämäläinen, Päivi
Hypoksian ja rauta-aineenvaihdunnan merkkiaineet metabolisessa oireyhtymässä ja suhteessa lipoproteiinipartikkelikokoon ja konsentraatioon
Kuopio: Itä-Suomen yliopisto
Publications of the University of Eastern Finland
Dissertations in Health Sciences 534. 2019, 75 s.
ISBN: 978-952-61-3207-5 (nid.)
ISSNL: 1798-5706
ISSN: 1798-5706
ISBN: 978-952-61-3208-2 (PDF)
ISSN: 1798-5714 (PDF)

TIIVISTELMÄ

Metabolisen oireyhtymän osatekijät: keskivartalolihavuus, kohonnut verenpaine, dyslipidemia ja kohonnut verensokeri muodostavat riskitekijäkasauman, joka lisää tyypin 2 diabetekseen ja sydän- ja verisuonitauteihin sairastumista ja kuolleisuutta. Metabolinen oireyhtymä on yleinen ja sen esiintyvyys kasvaa edelleen liittyen sekä väestön ikääntymiseen että ylipainon lisääntymiseen. Tarvitaan lisätietoa metabolisen oireyhtymän etenemiseen vaikuttavista tekijöistä ja toisaalta merkkiaineista, jotka voisivat helpottaa suuressa riskissä olevien potilaiden tunnistamista kliinisessä työssä.

Tämän tutkimuksen tavoitteena oli tutkia rasvakudoksen toimintahäiriön ja hypoksian sekä rauta-aineenvaihdunnan merkkiaineiden yhteyttä metaboliseen oireyhtymään ja sen osatekijöihin. Lisäksi tavoitteena oli selvittää aterogeenisen lipoproteiinipartikkeliprofiilin yhteyttä hemoglobiinitasoon. Erytropoetiinia, hemoglobiinia ja haptoglobiinia tutkittiin rasvakudoksen toimintahäiriön ja ferritiiniä ja transferriinireseptoria rauta-aineenvaihdunnan merkkiaineina poikkileikkaustutkimuksessa. Ferritiinitason muutoksen ja metabolisen oireyhtymän ja sen osatekijöiden kehittymisen tai parantumisen yhteyttä selvitettiin 6,5 vuoden seurantatutkimuksessa. Ydinmagneettiresonanssilla (NMR) mitatun lipoproteiinipartikkelien koon ja konsentraation yhteyttä hemoglobiinitasoon tutkittiin poikkileikkaustutkimuksessa. Tutkimusaineiston muodosti alunperin 1294 Pieksämäen kaupungin asukasta vuosina 1942, 1947, 1952 ja 1962 syntyneistä ikäkohorteista, jotka oli kutsuttu terveystarkastukseen vuosina 1997-1998 (lähtötilanne) ja uudelleen vuosina 2003-2004 (seuranta).

Tutkimuksessa todettiin, että metabolinen oireyhtymä on yhteydessä korkeampaan hemoglobiini-, erytropoetiini-, haptoglobiini ja ferritiinitasoon. Korkeampi hemoglobiini liittyy kaikkiin metabolisen oireyhtymän osatekijöihin, kun taas korkeampi erytropoetiinitaso on yhteydessä vain keskivartalolihavuuteen. Korkeampi ferritiinitaso liittyy dyslipidemiaan, keskivartalolihavuuteen ja kohonneeseen verensokeriin. Ferritiinitason nousu 6,5 vuoden seurannassa liittyy metabolisen oireyhtymän kehittymiseen sekä miehillä että naisilla. Ferritiinitason muutos on yhteydessä hyperglykemian, hypertriglyseridemian ja keskivartalolihavuuden kehittymiseen tai paranemiseen 6.5 vuoden seurannassa. Korkeampi hemoglobiinitaso on yhteydessä suurempaan VLDL, pienempään LDL ja HDL partikkelikokoon ja suurempaan määrään kooltaan suurempia VLDL ja pienempiä HDL partikkeleja.

Yhteenvetona voidaan todeta, että erytropoetiinitaso voi toimia yhtenä merkkiaineena metaboliseen oireyhtymään liittyvästä rasvakudoksen toimintahäiriöstä. Hemoglobiini on yleisesti käytetty helposti saatavilla oleva laboratoriokoe, joka voi mahdollisesti täydentää metabolisia riskitekijöitä omaavan potilaan kardiovaskulaaririskin arviota. Ferritiinitaso voi toimia merkkiaineena metabolisen oireyhtymän tunnistamisessa ja seurannassa. Ferritiinitasoa tulkittaessa tulee kuitenkin huomioida määritykseen liittyvät rajoitukset muiden sairauksien suhteen ja tarvittaessa käyttää täydentäviä rauta-aineenvaihdunnan tutkimuksia.

Luokitus: QU 85.6, WD 200.5.I7, WD 210, WH 150, WH 190, WK 810, WK 880
Yleinen suomalainen asiasanasto: metabolinen oireyhtymä; aineenvaihdunta; dyslipidemia; hemoglobiini; merkkiaineet; rauta

ACKNOWLEDGEMENTS

This study was carried out at the Institute of Public Health and Clinical Nutrition in the University of Eastern Finland.

I express my deepest gratitude to my supervisor Professor Mauno Vanhala, M.D., PhD, who originally collected the data of the metabolic syndrome project in Pieksämäki, on which this study is based. I thank him for sharing his expertise and knowledge during the study and his patience in encouraging me to finish it.

I also express my gratitude to my supervisor, Docent Juha Saltevo, M.D., PhD, who firstly introduced this project to me, for his guidance and advice during the current study and also his encouragement to complete this study.

My special gratitude is directed to Hannu Kautiainen, B.A., for his remarkable statistical help and advice during this study. I am grateful for those meetings in Äänekoski that were essential for the completion of the original publications.

I am grateful for my co-author Professor Pekka Mäntyselkä, M.D., PhD., for your participation and knowledge in the original publications and encouraging me in the last steps of this study.

I sincerely thank the official reviewers of this thesis, Professor Lena Thorn, M.D., PhD. and especially Docent Jorma Lahtela, M.D., PhD. for a smooth review process and their encouraging comments and constructive criticism, which led to the clear improvement of my thesis.

I respectfully thank all my colleges and especially Docent Saara Metso, M.D., PhD, in the Unit of Endocrinology in Tampere University Hospital, for your support and encouragement to complete the study.

I am deeply thankful to my parents who taught me the importance of education and hard work and who have always supported me.

Finally, I want to thank my friends and life companion for supporting me during this project and most importantly, thank you for precious moments with you outside working life.

This study has been financially supported by the Department of Public Health and Clinical Nutrition at the University of Eastern Finland and Tampere University Hospital research funding.

Tampere, September 2019
Päivi Hämäläinen

LIST OF THE ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Hämäläinen P, Saltevo J, Kautiainen H, Mäntyselkä P, Vanhala M. Erythropoietin, ferritin, haptoglobin, hemoglobin and transferrin receptor in metabolic syndrome: a case control study. *Cardiovasc Diabetol*. 2012 Sep 27; 11: 116. doi: 10.1186/1475-2840-11-116.

II Hämäläinen P, Saltevo J, Kautiainen H, Mäntyselkä P, Vanhala M. Serum ferritin levels and the development of metabolic syndrome and its components: a 6.5-year follow-up study. *Diabetol Metab Syndr*. 2014 Oct 26;6(1):114. doi: 10.1186/1758-5996-6-114.

III Hämäläinen P, Saltevo J, Kautiainen H, Mäntyselkä P, Vanhala M. Hemoglobin level and lipoprotein particle size. *Lipids Health Dis*. 2018 Jan 10;17(1):10. doi: 10.1186/s12944-018-0655-2.

CONTENTS

ABBREVIATIONS

ALAT	Alanine aminotransferase
BMI	Body Mass Index
CAD	Coronary artery disease
CVD	Cardiovascular disease
EPO	Erythropoietin
FFA	Free fatty acids
GT	Gamma-glutamyl transferase
GWAS	Genome-wide association studies
HDL	High density lipoprotein
HIF	Hypoxia inducible transcription factor
HOMA-IR	Homeostasis model for assessment of insulin resistance
hsCRP	Highly Sensitive C-reactive protein
IDF	International Diabetes Federation
IDL	Intermediate density lipoprotein
IL-6	Interleukin six
IR	Insulin resistance
LDL	Low density lipoprotein
MetS	Metabolic syndrome
MHO	Metabolically healthy obese
MONW	Metabolically obese normal weight
NCEP/ ATPIII	National Cholesterol Education Program's Adult Treatment Panel
NAFLD	Non-alcoholic fatty liver disease
NMR	Nuclear Magnetic Resonance
VLDL	Very low-density lipoprotein
WC	Waist circumference
WHO	World Health Organization
TG	Triglycerides
TFR	Transferrin receptor
TNF	Tumor necrosis factor
UC	Ultracentrifugatio

1 INTRODUCTION

The components of metabolic syndrome (MetS): abdominal obesity, increased blood pressure, glucose intolerance and dyslipidemia constitute a clustering of cardiometabolic risk factors (Depres 2006, Eckel et al. 2005). Each component of MetS is an independent risk factor for cardiovascular disease, and as a whole, MetS is associated with a twofold increase in cardiovascular outcomes, fivefold risk for type 2 diabetes and 1.5-fold increase in all-cause mortality (Grundy et al. 2005, Mottillo et al. 2010). The prevalence of MetS is already high and is increasing with the aging of the population and the prevalence of obesity (Beltran-sanchez et al. 2013, Ervin 2009, Balkau 2000). More information is needed on the factors affecting the progression of MetS and its transition to cardiovascular disease as well as on the markers that could recognize subjects at high risk.

Recent research suggests that adipose tissue dysfunction plays an important role in the development of MetS (Grundy 2015). Reduced adipose tissue oxygenation and cellular hypoxia may be an underlying cause of adipose tissue dysfunction, contributing to metabolic changes, including the insulin resistance associated with abdominal obesity and MetS (Pasarica et al. 2009, Regazzetti et al. 2009, Wood et al. 2009). Hypoxia is a known stimulator of erythropoietin (EPO) production, and EPO is a stimulator of hemoglobin synthesis (Bunn 2013). Higher EPO concentrations have been associated with larger body fat mass (Reinhardt et al. 2016), as well as higher hemoglobin levels with insulin resistance and type 2 diabetes (Barbieri et al. 2001, Choi et al. 2003, Tulloch-Reid et al. 2004). Haptoglobin is a circulating glycoprotein whose expression has also been found in human white adipose tissue, more in visceral than in subcutaneous fat (Fain et al. 2004, Gamucci et al. 2012). Haptoglobin expression is increased in obesity and haptoglobin levels are related to the degree of adiposity (Chiellini et al. 2004).

In addition to adipose tissue, iron and its metabolism have emerged as an important mediator of glucose and lipid metabolism. It has been suggested that iron excess promotes the development of insulin resistance, type 2 diabetes and cardiovascular disease through increased oxidative stress as a consequence of the pro-oxidant properties of iron (Fernandez-Real et al. 2014). However, obesity is associated with iron deficiency (Zhao et al. 2015) and conflicting results have been found between iron stores and development of cardiovascular disease (Das De et al. 2015, Von Haehling et al. 2015, Suarez-Ortegon et al. 2018). Ferritin is a key regulator of iron homeostasis and an accepted clinical measure of body iron stores (Cook et al. 2003). Transferrin receptors (TFR) mediate cellular uptake of circulating iron, and the measurement of the serum TFR serves as an index of tissue iron deficiency (Kohgo et al. 2008). Elevated serum ferritin levels have been demonstrated to be associated with obesity and predict type 2 diabetes (Bao et al. 2012, Kunutsor et al. 2013, Zhao et al. 2012). The association between serum TFR levels and prevalent or incident diabetes has been conflicting (Bao et al. 2012, Kunutsor et al. 2013, Zhao et al. 2012).

Changes in major lipoprotein particles classes: very-low density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) concentrations and sizes, have been demonstrated in metabolic syndrome and in insulin resistance, which can influence the risk of cardiovascular disease in MetS (Frazier-Wood et al. 2011, Kathiresan et al. 2006, Wang et al. 2012, Mora et al. 2010, Austin et al. 1995, Fagot-Campgna et al. 1999, Fizelova et al. 2015, Krauss 2010, Jellinger et al. 2012). However, measurements of lipoprotein particle sizes and concentrations are not easily available in clinical work in patient risk assessment. Hemoglobin level is a routinely measured parameter that is also associated with high cardiovascular risk conditions such as insulin resistance and type 2 diabetes (Barbieri et al. 2001, Choi et al. 2003, Tulloch-Reid et al. 2004).

The following review of the literature outlines metabolic syndrome and its pathophysiology, focusing on adipose tissue dysfunction, iron metabolism and lipoprotein particles.

Our own study investigates associations between metabolic syndrome and adipose tissue dysfunction and hypoxia as well as iron metabolism markers in a population sample. Erythropoietin, hemoglobin and haptoglobin are selected markers for adipose tissue dysfunction and ferritin and TFR for iron metabolism. In addition, we investigated the association between hemoglobin level and lipoprotein particle size or concentration assessed by proton nuclear magnetic resonance (NMR) spectroscopy.

2 REVIEW OF THE LITERATURE

2.1 DEFINITION OF METABOLIC SYNDROME

Metabolic syndrome (MetS) is defined by a constellation of interconnected physiological, biochemical, clinical, and metabolic factors that directly increase the risk of atherosclerotic cardiovascular disease (CVD), type 2 diabetes, and all-cause mortality (Grundy et al. 2005, Wilson et al. 2005). MetS was initially treated as a concept rather than a diagnosis. Metabolic syndrome has its origins in 1920 when a Swedish physician demonstrated an association between high blood pressure (hypertension), high blood glucose (hyperglycemia), and hyperuricemia (gout) (Kylin, 1923). Later in 1947, a French physician, Vague, showed that visceral obesity was commonly associated with the metabolic abnormalities found in CAD and type 2 diabetes (Vague, 1947). Following this, in 1965, an abstract was presented at the European Association for the Study of Diabetes annual meeting describing a syndrome which comprised hypertension, hyperglycemia, and obesity (Avogaro et al. 1965). The field moved forward significantly following the 1988 Banting Lecture given by Gerald Reaven (Reaven, 1988). Reaven described a cluster of risk factors for diabetes and cardiovascular disease and named it "Syndrome X". His main contribution was an introduction of the concept of insulin resistance. In 1989, the syndrome was renamed "the deadly quartet" for the combination of upper body obesity, glucose intolerance, hypertriglyceridemia, and hypertension (Kaplan, 1989) and later, in 1991, it was again renamed "the insulin resistance syndrome" (DeFronz, 1991).

The term metabolic syndrome has become widely used since the first internationally accepted criteria were proposed by a diabetes consultation panel for the World Health Organization (WHO) in 1998 (Alberti et al. 1998). The WHO definition was based on insulin resistance (defined by hyperinsulinemia, impaired glucose tolerance (IGT) or type 2 diabetes as a basic component). At least two of the additional factors (obesity, hypertriglyceridemia, low HDL cholesterol levels, hypertension and microalbuminuria) were needed to fulfill the MetS criteria in WHO definition (Table 1). In practice, insulin resistance in the WHO definition was difficult to use. The oral glucose tolerance test (OGTT) was required, and among subjects with normal glucose tolerance, insulin resistance was proven with the expensive and time-consuming glycemic clamp technique. Furthermore, the WHO definition was criticized because of the inclusion of microalbuminuria in the criteria, while there was no consensus on the association of microalbuminuria with insulin resistance (Jager, 1998).

In order to facilitate the clinical and epidemiological application of MetS, the National Cholesterol Education Program's Adult Treatment Panel III (NCEP, ATPIII) proposed a new definition in 2001 based on five measurements and laboratory results: waist circumference, triglycerides, HDL-cholesterol, blood pressure, and glucose (NCEP, 2002). Central obesity was determined in this definition with sex-specific limits of waist circumference instead of the waist-to-hip ratio, which was used in the WHO definition. Microalbuminuria was removed from the criteria as well as insulin resistance, which was a notable difference compared to the WHO definition (Table 1).

The NCEP/ATPIII definition was followed by the International Diabetes Federation (IDF) definition in 2005, which highlighted abdominal obesity as a key factor for the development of MetS and considered its presence mandatory (Alberti et al. 2006). However, a single definition and the contributions of the underlying components of MetS have been of much debate over the decades. The commonality among the different definitions is that each recognizes components of obesity or abdominal adiposity or insulin resistance, impaired glucose metabolism, hypertension, and atherogenic dyslipidemia.

In 2009 a joint statement regarding the harmonization of the criteria was released. This harmonized definition stated that obesity and insulin resistance are not pre-requisites for MetS but that three of the five components would suffice for a diagnosis of MetS, with the thresholds for measuring waist circumference (WC) requiring ethnic and nation specificity (Alberti et al. 2009). The harmonized definition highlights the need for more study and evidence to determine the WC cutoffs in different populations that are associated with higher risk, recognizing that the relationship between abdomen obesity and the risk of T2DM or CVD differs in different populations (Alberti et al. 2009). The higher threshold for Caucasian WC (102 cm in men and 88 cm in women) was kept for North American patients, but it was acknowledged that lower values

used by the earlier IDF definition could be important for those at higher risk. The definition leaves to clinical judgment regarding patients with mixed ethnicity.

According to these harmonized criteria, a subject with three or more of the following components can be classified as having MetS: 1) increased waist circumference (≥ 102 cm (≥ 40 in) for men and ≥ 88 cm (≥ 35 in) for women in Caucasian population); 2) elevated fasting total triglycerides (≥ 1.7 mmol/l (≥ 150 mg/dl) or treatment for dyslipidemia); 3) low fasting serum high density lipoprotein (HDL) cholesterol (<1.03 mmol/l (<40 mg/dl) in men or <1.29 mmol/l (<50 mg/dl) in women or treatment for dyslipidemia); 4) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg or the use of antihypertensive medication; and 5) fasting plasma glucose ≥ 5.6 mmol/l (≥ 100 mg/dl) or the use of antihyperglycemic medication (Table 1).

Table 1. Comparison of four selected definitions of MetS.

Definition	**WHO** (Alberti et al. 1998)	**NCEP/ATPIII** (NCEP, 2002)	**IDF** (Alberti et al. 2006)	**Harmonized definition of NCEP/ATPIII** (Alberti et al. 2009)
Year	1998	2001	2005	2009
Number of risk factors	IFG or IGT or T2DM or lowered insulin sensitivity* and 2 of following:	Three or more of following:	Obesity and 2 of following:	Three or more of following:
Obesity	Waist/hip ratio >0.9 M, >0.85 F or BMI >30kg/m^2	WC ≥102 cm M ≥88 cm F	WC ≥ 94 cm M ≥ 80 cm F	WC Geographic and ethnic specific
Dyslipidemia	HDL-C <0.91mmol/l M <1.0 mmol/l F TG ≥ 1.7mmol/l	HDL-C <1.0 mmol/l M < 1.3mmol/l F TG ≥ 1.7mmol/l	HDL-C < 1-0 mmol/l M < 1.3 mmol/l F TG ≥ 1.7mmol/l or medication	HDL-C < 1-0 mmol/l M < 1.3 mmol/l F TG ≥ 1.7mmol/l or medication
Hyperglycemia	T2DM or FPG >6.1 mmol/l or 2h OGT >7.7 mmol/l	T2DM or FPG ≥6.1 mmol/l	T2DM or FPG ≥5.6 mmol/l	FPG ≥5.6 mmol/l or medication
Hypertension	SBP ≥ 140 mmHg DBP ≥ 90 mmHg	SBP ≥ 130 mmHg DBP ≥ 85 mmHg	SBP ≥ 130 mmHg DBP ≥ 85 mmHg or medication	SBP ≥ 130 mmHg DBP ≥ 85 mmHg or medication
Other	Microalbuminuria: Urinary excretion rate of >20 μg/min or albumin:creatinine ratio of >30 mg/g			

BMI, body mass index; DBP, diastolic blood pressure in mmHg; F, female; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; IDF, the International Diabetes Federation; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; M, male; OGT, oral glucose tolerance test; NCEP/ATPIII, the National Cholesterol Education Program's Adult Treatment Panel III, SBP, systolic blood pressure in mmHg; TG, triglyceride; WC, waist circumference; WHO, World Health Organization.
* Insulin sensitivity measured under hyperinsulinemic euglycemic conditions, glucose uptake below lowest quartile for background population under investigation.

2.2 EPIDEMIOLOGY OF METABOLIC SYNDROME

The reported prevalence of MetS varies depending on the definition used, age, sex, socioeconomic status, and the ethnic background of study cohorts (Table 2). However, it is generally accepted that the prevalence of MetS is increasing, in accordance with increasing body mass index (BMI) and age. In the United States, MetS prevalence increased from 29 to 34 % between years 1988-1994 and 1999-2006 in the National Health and Nutrition Examination Survey (NHANES) according to the NCEP/ATPIII definition (Mozumbar et al. 2011).

In Europe, in a Danish study published in 2007 including 2,493 participants aged 41–72, the NCEP: ATPIII definition identified 18.6% of males and 14.3% of females as having MetS (Jeppesen et al. 2007). In Finland, according to modified WHO criteria MetS was present in 39% of the men and 22% of the women aged 45-64 years in a population-based sample of 2,049 individuals in 2004 (Ilanne-Parikka et al. 2004). In 2010, the

population-based Health 2000 Study in Finland included 6,105 individuals, aged 30–79 and identified MetS according to IDF, NCEP: ATPIII and the new harmonized criteria of 2009. The highest prevalence estimates of MetS were observed with the harmonized definition: 47.8% in men and 40.7% in women (Pajunen et al. 2010).

Table 2. Prevalence of Metabolic Syndrome in different populations.

Population	MetS prevalence (Year)	MetS definition	Population demographic
United States (Mozumbar et al. 2011) (Aguilar M et al. 2015)	29% (1988-94) 34% (1999-2006) 33-39% (2003-2012)	NCEP:ATPIII (2001)	Age >20 years
Europe Finland, Netherlands, United Kingdom, Sweden, Poland, Italy (Gao et al. 2008)	41% men 38% women (1990, Finland 2002)	IDF	Age 47-71 years
Korean (Lee et al. 2013)	25% (1998-2008)	NCEP:ATPIII (2001) WC ≥ 90cm men WC ≥ 85cm women	Age >20 years
Japan (Unno et al. 2012)	36% men 10% women (2009)	NCEP:ATPIII (2001) WC ≥ 85cm men WC ≥ 90cm women	Age 30-69 years
China (Gu et al. 2005)	10% men 18% women	NCEP:ATPIII (2001) WC ≥ 90cm men WC ≥ 80cm women	Age 30-74 years Prevalence higher in urban areas
Finland (Ilanne-Parikka et al. 2004) (Pajunen et al. 2010)	38% men 22% women (2004) 48% men 41% women (2000-2007)	WHO Harmonized definition	Age 25-65 years Age 30-79 years

IDF, the International Diabetes Federation; NCEP/ATPIII, the National Cholesterol Education Program's Adult Treatment Panel III; WHO, World Health Organization

2.3 CARDIOVASCULAR, DIABETES AND ADDITIONAL DISEASE RISK IN METABOLIC SYNDROME

MetS can identify patients with a high risk of cardiovascular disease (CVD) as well as type 2 diabetes. Subjects with MetS have been indicated to have a twofold increased risk of cardiovascular events (Grundy et al. 2005, Mottillo et al. 2010). The Botnia study, which involved 4,483 middle-aged participants in Finland and Sweden, showed a marked increase from 2 to 12% in cardiovascular mortality in participants with metabolic syndrome during a 6.9-year follow-up period (Isomaa et al. 2001). In the Kuopio Ischemic Heart Disease Risk Factor Study, metabolic syndrome was associated with a 2.5 to 2.8-fold greater risk of death from any cardiovascular cause (Lakka et al. 2002). In a meta-analysis of 87 studies (n = 951,083) metabolic syndrome was associated with a 2.3-fold increase in the risk for CVD, 2.4-fold risk for CVD mortality, 1.6-fold risk in all-cause mortality, 2-fold risk for myocardial infarction and 2.3-fold risk for stroke (Mottillo et al. 2010). After synthesizing the results of the studies conducted in patients without type 2 diabetes mellitus, metabolic syndrome remained associated with high cardiovascular risk, ranging from 1.6-fold for myocardial infarction to a 1.9-fold risk for stroke (Mottillo et al. 2010) suggesting that metabolic syndrome maintains its prognostic value for cardiovascular outcomes in the absence of type 2 diabetes. However, it should be taken into consideration that MetS does not include many of the factors that determine absolute CVD risk like age, sex, smoking and low-density lipoprotein (LDL) cholesterol levels (Alberti et al. 2009).

Although the original intent for defining MetS was to identify subjects at risk for CVD, MetS also confers a 5-fold increase in risk for type 2 diabetes mellitus (Alberti et al. 2009). In the Framingham Heart Study

Offspring, participants' MetS was associated with a 7-fold age-adjusted risk for diabetes in males and females (Wilson et al. 2005, Wilson et al. 2008). A meta-analysis with 42,419 participants from 16 cohorts found the relative risk of an incidence of type 2 diabetes to be 3.5–5.2 times higher, with no significant differences in the definition of MetS used (Ford et al. 2008a).

Additional disease risk can be considered to be associated with MetS in relation to the component of abdominal obesity. Twenty percent of all cancer deaths in women and 14% in men can be attributed to obesity (Gilbert et al. 2013). There are data showing that there is an increased risk of colon, kidney, prostate, endometrial, and breast cancer with obesity (Giovannucci et al. 2010). The underlying reason behind these observations remains to be elucidated, but possibilities are promotion of cancer growth by adipose tissue inflammation, hyperglycemia or hyperinsulinemia (Giovannucci et al. 2010). In a meta-analysis of 87 studies (n = 951,083) metabolic syndrome was associated with 1.6-fold risk in all-cause mortality (Mottillo et al. 2010).

MetS and the abdominal obesity component are also associated with nonalcoholic fatty liver disease (NAFLD). The risk factors for the development of NAFLD are aging, a high energy diet high in saturated fat, and obesity; the prevalence of NAFLD has increased with increasing obesity (Krawczyk et al. 2010). NAFLD is characterized by an elevated hepatic fat content in the absence of hereditary or secondary causes of fat accumulation in the liver (Chalasani et al. 2017). NAFLD encompasses a continuum of pathologic changes to the liver from steatosis to steatohepatitis, which can further develop into cirrhosis and increased risk of hepatocellular carcinoma (Krawczyk et al. 2010, Starley et al. 2010). NAFLD has sometimes been referred to as MetS of the liver, and there has been some discussion as to whether this could be included as a MetS component as a hepatic element of insulin resistance (Yki-Järvinen 2014, Smits et al. 2013). NAFLD is associated with MetS components of increased waist circumference, triglycerides, hypertension, hyperglycemia, and lower HDL levels, and the prevalence of NAFLD increases with the number of MetS components present (Smits et al. 2013). The metabolic disorders associated with NAFLD are mainly dyslipidemia and type 2 diabetes (Chalasani et al. 2017). However, a significant proportion of NAFLD patients do not have MetS and statistical modeling has not supported the idea that NAFLD is an independent manifestation that should be added as a component of MetS (Smits et al. 2013). Nonetheless, a diagnosis of MetS could mean that NAFLD is present as an additional risk factor.

2.4 COMPONENTS OF METABOLIC SYNDROME

2.4.1 Abdominal obesity

The worldwide increase in the prevalence of obesity, defined as BMI > 30 kg/m^2, is an important underlying cause for the increasing prevalence of MetS (Mozumbar et al. 2011). In Finland, in the National FinTerveys 2017 survey, among a Finnish population aged 18-64 years, 25% of men and 24% of women were obese (Koponen et al. 2017). A waist circumference that was greater than normal (>100 cm for men and > 90 cm for women) was noted in 36% of men and 35% (Terveys 2017). Basically, obesity results from an imbalance between energy intake and energy expenditure. The causes of weight gain are natural consequences of the westernized life style, with its excess of energy together with a sedentary lifestyle (Ford et al., 2008b). Alterations of lifestyle probably explain obesity worldwide, but the cause of individual obesity also involves genetic, environmental and psychosocial factors (Choquet et al. 2011, Symons et al. 2011). Obesity-associated diseases and metabolic disturbances are thus presumed to result from and be modified by gene-environment interaction (Choquet et al. 2011, Symons et al. 2011).

While BMI provides an indicator of overall obesity for epidemiological purposes, it has a limited ability to identify subjects at a high risk of CVD or type 2 diabetes. It has been found that there exist metabolically obese, normal-weight (MONW) subjects: individuals who have normal BMI values but who nonetheless suffer from metabolic complications commonly found in obese people (St-Omge et al. 2004). Conversely, metabolically healthy obese (MHO) individuals have a BMI above 30 kg/m^2 but are not characterized by insulin resistance or dyslipidemia (Karelis et al. 2004). A key factor underpinning the difference in CVD risk between MONW and MHO subjects is the likely presence of excess visceral adipose tissue (Fujioka et al. 1987, Tai et al. 2000). Most MONW individuals with relatively low BMI likely have a significant excess of visceral adipose tissue, and most MHO individuals with a high BMI likely have much less visceral adipose tissue (Ruderman et al. 1998). It has been demonstrated that very obese individuals with a small

amount of visceral adiposity, active sumo wrestlers, for example, are quite insulin sensitive, whereas retired sumo wrestlers with greater amounts of visceral adipose tissue tend to have insulin resistance, dyslipidemia, and a high prevalence of metabolic complications such as type 2 diabetes and CVD (Matsuzawa 1997).

Upper body adipose tissue consists of intra-abdominal and abdominal subcutaneous fat depots. Intraperitoneal fat is visceral adipose tissue that is associated with the digestive organs, and include the omental (associated with the stomach), the mesenteric (associated with the intestine), and epiploic (along the colon) (Shen W et al., 2003). Abdominal obesity, particularly visceral obesity, but also including fat accumulation in the abdominal subcutaneous area, confers an increased risk for metabolic complications of obesity, whereas lower or peripheral obesity, preferential fat accumulation in the gluteofemoral region and leg is associated with lower risk and may be protective (Azuma et al., 2007, Fox et al., 2007, Vega et al., 2006). Waist circumference (WC) is a clinical measure of abdominal obesity. It measures both visceral and subcutaneous abdominal fat depots. The amounts of visceral fat increase progressively through each category of increasing WC although WC can be more strongly correlated with subcutaneous abdominal fat area (Grundy et al. 2013). The most reliable assessment of abdominal fat distribution can be obtained with computed tomography (Chowdhury et al. 1994) or magnetic resonance imaging (Ross et al. 1992). However, a high WC is clearly associated with metabolic risk factors (Alberti et al. 2009). Waist circumference has been shown to be a better predictor of insulin resistance, type 2 diabetes and CVD than waist-hip ratio or BMI (Balkau et al. 2007, Karter et al. 2005, Wang et al. 2005).

2.4.2 Dyslipidemia

The dyslipidemic state frequently observed in patients with abdominal obesity is a key feature of the clustering abnormalities of the metabolic syndrome (Despres et al. 1990, Grundy et al. 2005). It includes high levels of triglycerides, low levels of high-density lipoprotein (HDL) cholesterol, relatively normal total and low-density lipoprotein (LDL) cholesterol levels, but more LDL particles that are smaller than normal (Despres et al. 1990, Grundy et al. 2005). In a typical clinical setting, hypertriglyceridemia and low HDL cholesterol will, therefore, be the two major dyslipidemic abnormalities associated with abdominal obesity and metabolic syndrome.

An increased proportion of small, LDL and HDL particles are important aspects of the dyslipidemic state in abdominal obesity (Pascot et al. 2001, Tchernof et al. 1996). This phenomenon is due to the remodeling of these lipoproteins in the circulation by the enzymes cholesteryl ester transfer protein and hepatic triglyceride lipase (Taskinen 2003, Taskinen 2005). Lipid exchanges by cholesteryl ester transfer protein have been shown to be largely driven by the concentration of triglyceride-donor lipoproteins, especially very low-density lipoproteins (VLDL) (Eisenberg 1984). Thus, in the presence of hypertriglyceridemia, an elevated concentration of large VLDL particles promotes the transfer of triglyceride molecules to LDL and HDL in exchange for cholesteryl ester molecules. As a consequence, both triglyceride-enriched LDL and HDL particles of viscerally obese patients become good substrates for hepatic triglyceride lipase, leading to the depletion of the lipid core of these lipoproteins, thereby forming small LDL and HDL particles (Eisenberg 1984, Lamarche et al. 1999). Smaller HDL have reduced cholesteryl ester core content and become more sensitive to degradation and increased clearance from the blood. This phenomenon partly explains the low HDL cholesterol levels frequently found in individuals with abdominal obesity (Lamarche et al. 1999).

The combination of high triglyceride, low HDL cholesterol levels and small, dense LDL particles has been termed the "atherogenic lipid triad"; it has been recognized as a major CVD risk factor (Austin et al. 1990, Grundy, 1998). Dyslipidemia linked to abdominal and visceral obesity is a major CVD risk factor and represents one of the abnormalities upon which the definition of the metabolic syndrome is based (Tchernof et al. 2013).

2.4.3 Hyperglycemia

Most persons with the metabolic syndrome have elevated fasting plasma glucose (Grundy 2012). The primary cause of hyperglycemia in patients with metabolic syndrome is insulin resistance (Grundy 2012). However, otherwise normal persons who are insulin resistant can avoid elevated glucose levels with

compensatory hyperinsulinemia. When pancreatic beta cell function begins to decline, glucose levels start to rise. Thus, hyperglycemia typically is not the first indication of metabolic syndrome, but develops later as a result (Grundy 2015). Impaired insulin action in the adipose tissue results in increased breakdown of lipids and increased flux of free fatty acids (FFA) from adipocytes to peripheral tissues, which also inhibits insulin signaling and further worsens insulin resistance (Lewis et al. 2002, Samuel et al. 2012). With hepatic insulin resistance and an abundance of FFA substrate, gluconeogenesis is increased, contributing to hyperglycemia (Lewis et al. 2002, Samuel et al., 2012). Myocellular insulin resistance also results in decreased glucose disposal peripherally (Boden et al. 2001). Over time, pancreatic beta cells continue to decompensate for the increased need for insulin to overcome resistance, and type 2 diabetes is the consequence (Boden et al., 2001).

2.4.4 Hypertension

Essential hypertension is frequently associated with several metabolic abnormalities, of which obesity, glucose intolerance, and dyslipidemia are the most common (Ferrannini et al. 1991). Studies suggest that both hyperglycemia and hyperinsulinemia activate the renin angiotensin system by increasing the expression of angiotensinogen, angiotensin II, and the angiotensin receptor, which, in concert, may contribute to the development of hypertension in patients with insulin resistance (Malhotra et al. 2001). There is also evidence that insulin resistance and hyperinsulinemia lead to sympathetic nervous system activation, and, as a result, the kidneys increase sodium reabsorption, the heart increases cardiac output, and arteries respond with vasoconstriction, resulting in hypertension (Morse et al. 2005). It has been also discovered that adipocytes produce aldosterone in response to angiotensin II (Briones et al. 2012).

The pathophysiologic mechanisms of metabolic syndrome are complex and are still not fully understood. In addition, it is even unclear whether the individual components of MetS are separate pathologies or manifestations with a common pathogenic mechanism.

However, it is generally accepted that several factors affect the development of MetS. These factors have been divided previously into three categories: 1) obesity and adipose tissue, 2) insulin resistance, and 3) independent factors such as molecules of hepatic, vascular, and immunologic origin, which can mediate components of MetS (Grundy et al. 2004). The most widely accepted and earliest hypothesis for the underlying pathophysiology of metabolic syndrome is that of insulin resistance (Reaven 1988, Reaven 2011, Eckel et al. 2005). However, obesity and particularly the excess and dysfunction of adipose tissue has been demonstrated to be an important trigger for most of the pathological pathways involved in MetS including insulin resistance (Yang et al. 2012, McLaughlin et al. 2007, Bluher et al. 2013, Cao et al. 2013).

2.5 PATHOPHYSIOLOGY OF METABOLIC SYNDROME

2.5.1 Insulin resistance

Insulin resistance (IR) is a pathological state of inadequate cellular response to insulin in cells that are insulin-dependent such as adipocytes, hepatocytes and skeletal muscle cells. IR is inversely correlated to insulin sensitivity and disables a tissue's ability to take up and utilize glucose, the preferred metabolic substrate (Perry et al. 2014). Normally after carbohydrates are ingested, insulin increases glucose transport and glycogen synthesis in the skeletal muscle cells. In the liver, insulin also promotes glycogen synthesis and de novo lipogenesis while inhibiting gluconeogenesis (Samuel et al. 2012). In the adipose tissue, insulin suppresses lipolysis and induces fatty acid uptake from circulating lipoproteins by stimulating activity of the hydrolyzing enzyme, lipoprotein lipase. Insulin also increases the storage of triglycerides in adipose tissue (Kahn et al. 2000).

In an insulin-resistant state, insulin signaling is impaired, resulting in decreased skeletal muscle glucose uptake and increased glucose transition to the liver. In the liver, the regulation of gluconeogenesis and glycogen synthesis is impaired and the formation of lipids is increased (Samuel et al. 2012). Adipocytes are the most insulin-dependent cells in obese humans. Impaired insulin action in the adipose tissue results in increased breakdown of lipids and increased flux of free fatty acids (FFA) from adipocytes, which will promote re-esterification of lipids in other tissues and further worsen insulin resistance (Lewis et al. 2002,

Samuel et al, 2012,). The increased FFA flux from adipose tissue to non-adipose tissue contributes to an increased hepatic synthesis of triglycerides and very-low-density lipoproteins (VLDL) (Adeli et al. 2001). This FFA flux plays an important role in the progression from normal glucose tolerance to fasting hyperglycemia (Adeli et al. 2001, Lewis et al. 2002, Ravussin et al. 2002). Together these changes can lead to the development of hyperglycemia and atherogenic dyslipidemia.

2.5.2 Genetic factors

Genetic factors predispose to MetS and its components together with environmental and behavioral factors. Genetic approaches that can be used for the discovery of genes associated with MetS are genome-wide associating studies (GWAS) and the candidate gene approach (Ziki et al. 2017).

GWAS examine the genome for common polymorphism associated with the disease and are suitable for complex traits (Ziki et al. 2017). Nonetheless, GWAS have several limitations. Uncovered genetic variants are not necessarily causative, the inherent effect of common variants is small and their association with more than two metabolic traits have been weak (Ziki et al. 2017). However, a number of gene variants have been identified by GWAS that have provided insight especially into obesity and metabolic syndrome-associated traits (Claussnitzer et al. 2015, Loche 2015, Shungin 2015, Yaghootkar et al. 2014).

One of the obesity-associated genes discovered in GWAS has been the FTO gene (fat mass and obesity associated gene). Pathogenic alleles of the FTO gene have been shown to shift adipocyte differentiation from beige (energy dissipating cells) to white (energy storing) adipocytes (Claussnitzer et al. 2015). Some of the obesity-associated gene loci have shown evidence of association with other traits of MetS, such as higher triglycerides, lower HDL, increased blood pressure and type 2 diabetes (Loche 2015, Shungin 2015). Genetic risk scores using 19 variants were associated with insulin resistance from prior GWAS studies; patients with more than 17 at-risk alleles were at a significantly increased risk for type 2 diabetes and CAD compared to those with less than 9 at-risk alleles (Yaghootkar et al. 2014). An analysis of genetic risk scores showed that 11 variants out of the 19 were associated with higher TG, lower HDL and greater hepatic steatosis (Yaghootkar et al. 2014).

One mechanism by which obesity-associated gene loci could influence obesity is alternative splicing, which is an essential regulatory mechanism for the generation of transcript. Aberrant splicing has been linked to obesity and insulin resistance (Pihlajamäki et al. 2011). Differential splicing of specific genes in obesity-associated loci has been found between overweight type 2 diabetes patients and lean normoglycemic individuals (Kaminska et al. 2016). Also, differential splicing of obesity-associated genes was found between visceral and subcutaneous fat (Kaminska et al. 2016).

Mutation burden analysis of the candidate genes was among the first methods used for discovering MetS-associated genes. Its approach aims to identify genes on the basis of information about their function. The majority of the identified disease genes underlie only one metabolic trait and most genetic associations failed to be replicated (Ziki et al. 2017). A few exceptions include variations in the adiponectin gene, ADIPOQ, associated with type 2 diabetes, hypertension and dyslipidemia (Lu et al. 2014, Vaxillaire et al. 2008,).

2.5.3 Adipose tissue dysfunction

Obesity is a clinical indicator of a state of overnutrition. Excess body fat is recognized as a heterogeneous condition in which individuals with similar levels of body mass index may have a different metabolic profile (Despres 2012). Waist circumference is a measure of abdominal obesity and body fat distribution, which is part of the definition of metabolic syndrome. Abdominal obesity consists of visceral adipose tissue located inside the peritoneum and around internal organs and subcutaneous adipose tissue located under the skin. Adipocytes in visceral adipose tissue are metabolically more active than cells in subcutaneous adipose tissue. Studies have demonstrated that increased visceral adipose tissue is more strongly associated with negative outcomes of obesity including insulin resistance and metabolic syndrome than subcutaneous adiposity (Wagenknecht et al. 2003, Fox et al. 2007).

Adipose tissue is an active endocrine organ that produces and regulates endocrine and paracrine hormones called adipokines (Dahlman et al. 2012, Lehr et al. 2012). Adipokines that are classified as anti-inflammatory and pro-inflammatory peptides have been shown to be associated with insulin resistance

and lipid metabolism in patients with obesity (Dahlman et al. 2012, Lehr et al., 2012 Voorde et al. 2013). The unbalanced production of pro- and anti-inflammatory adipokines in visceral obesity can contribute to the development of metabolic syndrome (Hotamisligil 2006).

During obesity and energy overbalance, hyperplasia and hypertrophy of adipocytes leads to adipose tissue expansion. A study of healthy adults was able to show that eight weeks of overfeeding increased the number of adipocytes in association with the enlargement of the fat depot (Tchoukalova et al. 2010). Adipocyte hypertrophy creates areas of local adipose tissue hypoxia at the earliest stages of expansion (Trayhurn et al. 2004).

Clinical observations in humans suggest that adipose tissue is poorly oxygenated in the obese state (Virtanen et al. 2002). If angiogenesis does not follow the adipose tissue expansion, a regulator of hypoxia and oxygen homeostasis, hypoxia-induced transcription factor (HIF) expression, is increased (Kaelin et al, 2008, Rupnick et al. 2002). As a result of this aberrant HIF expression, white adipose tissue accumulates fibrillar collagens, resulting in local fibrosis. Additionally, HIF stimulates macrocyte-related inflammatory gene expression, leading to macrophage recruitment and inflammation in adipose tissue (Divoux et al. 2012, Jang et al. 2016, Pasarica et al. 2009b). Under hypoxia, infiltrated macrophages express a repertoire of proinflammatory factors characteristic of insulin resistance (Sun et al. 2011). Thus, hypoxia-induced fibrosis in adipose tissue may be a key factor that stimulates the local inflammatory responses and results in insulin resistance (Halberg et al. 2009, Pasarica et al. 2009a, Regazzetti et al. 2009).

Hypoxia also dysregulates the production of many inflammation-related adipokines, such as interleukin six (IL-6), leptin and adiponectin (Hosogai et al. 2007, Ye et al. 2006).

2.6 MARKERS OF ADIPOCYTE DYSFUNCTION AND HYPOXIA

2.6.1 Hemoglobin

Erythrocytes deliver oxygen to all the tissues in the body. Most of the cytoplasmic protein of the erythrocytes consist of hemoglobin, iron-containing molecules that have the ability to combine with and carry oxygen molecules (Rose et al. 1998). Hemoglobin synthesis follows erythrocytes differentiation and production. Most of the iron in the body, from 60 to 75 per cent, is incorporated in hemoglobin, and adequate availability of iron is essential for normal hemoglobin and red blood cell production (Andrews, 1999). Erythropoietin (EPO) is the primary regulator of erythrocytes production (Bunn 2013, Krantz 1991).

Hematological parameters: red blood cell count, volume percentage of red blood cells (hematocrit) and hemoglobin have been shown to be independently associated with insulin resistance (Barbieri et al. 2001, Choi et al. 2003). Also, higher hemoglobin concentrations have been present in individuals with metabolic syndrome or type 2 diabetes versus healthy controls or obese subjects versus non-obese subjects (Tulloch-Reid et al. 2004, Arakaki et al. 2016, Lohsoonthorn et al. 2007, Laudisio et al. 2013). Also, previously, increasing hemoglobin levels were associated with increasing arterial stiffness, which is a predictor of morbidity and mortality in CVD in high-risk populations (Kawamoto et al. 2012).

The mechanism that could explain the association between insulin resistance, type 2 diabetes or CVD risk and elevated hemoglobin levels is not completely understood. Firstly, insulin has a synergistic effect together with erythropoietin on stimulating erythrocytes production and the role of insulin in the regulation of human erythropoiesis has previously been documented (Bersch et al. 1982). Thus, hyperinsulinemia in insulin resistance could promote erythrocytosis. Secondly, hemoglobin regulates endothelial function by modulating the bio-availability of nitric oxide at the tissue level and hemoglobin level has been inversely associated with vascular endothelial function in type 2 diabetes patients (Sonmez et al. 2010). Additionally, a higher level of hemoglobin is inversely associated with the level of adiponectin, one of the adipokines, which regulates lipid and glucose metabolism, and is inversely associated with obesity and the amount of visceral adipose tissue (Kawamoto et al., 2011). Furthermore, a higher hemoglobin level is associated with higher levels of pro-inflammatory cytokines produced in the adipose tissue of obese prediabetic subjects (Kutlu et al. 2009). In addition, visceral adiposity has been associated with hemoglobin level and the association between hemoglobin level and insulin resistance have been dependent on the level of visceral adiposity (Tabara et al. 2013).

Thus, visceral adiposity is at least one of the underlying factors for the associations observed between hemoglobin and insulin resistance and type 2 diabetes.

2.6.2 Haptoglobin

Haptoglobin is a classic acute phase glycoprotein in human plasma that is expressed in many tissues and cell types, but the liver is the quantitative major source. Haptoglobin has an important binding function of hemoglobin during hemolysis when hemoglobin escapes the intracellular compartment of red cells (Andersen et al. 2012). Under normal physiological conditions, hemoglobin is bound and stabilized by haptoglobin and subsequently cleared from circulation by the macrophage-specific receptor (Kristiansen et al. 2001). Haptoglobin concentration can increase during inflammation in response to cytokines like interleukin-6 (Levy et al. 2010). It has been found that also human white adipose tissue expresses haptoglobin (Fain et al. 2004a, Fain et al. 2004b). A higher haptoglobin production is found in the visceral and omental fat tissue compared to the subcutaneous depot (Gamucci et al. 2012, Fain et al. 2010). Obesity is associated with inflammation and macrophage infiltration in white adipose tissue and haptoglobin has been found to be one of the chemotactic molecules that is involved with macrophages recruitment (Maffei et al. 2009). In turn, inflammation and cytokines are possible triggers of haptoglobin production in white adipose tissue (Friedrics et al. 1995). Haptoglobin expression in adipocytes is increased in obesity and circulating haptoglobin levels have been positively correlated with body mass index and the level of adiposity (Chiellini et al. 2004, Friedrics et al. 1995).

2.6.3 Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone that is one of the primary regulators of red blood cell production (Bunn 2013, Krantz et al. 1991, Jelkmann 1992). In bone marrow, EPO promotes the proliferation of erythroid progenitor cells and increases the production of red blood cells and thereby hemoglobin synthesis (Bunn 2013, Krantz et al. 1991, Jelkmann 1992). EPO is a mediator of the hypoxic induction of erythropoiesis (Bunn 2013). Hypoxia induces an increase in erythropoietin production in the kidneys, which is the main site of EPO synthesis in adult, but the liver, too, can be stimulated under hypoxia and contribute to plasma EPO levels (Koury et al. 2015).

Hypoxic induction of erythropoietin synthesis depends in large part on the hypoxia-inducible transcription factor (HIF) pathway, which is activated in virtually all cells by exposure to hypoxia (Wan et al. 1993). Hypoxia results in increased transcription of HIF-regulated genes including erythropoietin and erythropoietin receptor (Kaelin etl al. 2008). HIF has been reported to have hundreds of target genes that regulate not only erythropoiesis but also iron metabolism, glucose and lipid metabolism, inflammation and angiogenesis (Koivunen et al. 2016). Some target genes are more specific for HIF1 whereas HIF2 is the main driver of transcription of erythropoietin.

Increasing evidence has suggested that reduced adipose tissue oxygenation and cellular hypoxia may be an underlying cause of adipose tissue dysfunction, contributing to metabolic changes associated with obesity and metabolic syndrome (Pasarica et al. 2009, Regazzetti et al. 2009, Wood et al. 2009). Erythropoietin receptors, specific cell surface receptors for erythropoietin, are expressed also in non-hematopoietic cells including white adipose tissue (Teng et al. 2011, Rankin et al. 2012).Additionally, over-expression of erythropoietin gene transcription stimulating factor (HIF) has been shown in the adipose tissue of obese subjects (Regazzetti et al. 2009, Wood et al. 2009).

Higher erythropoietin concentrations have been associated with higher fat body mass in a study of the Pima Indians of Arizona, a population that has a high prevalence of obesity and type 2 diabetes (Reinhardt et al. 2016). In addition to obesity, previous studies have found associations between higher erythropoietin concentrations and higher cardiovascular risk. In a study of renal transplant recipients, higher baseline endogenous erythropoietin concentrations were associated with higher cardiovascular mortality independent of other risk factors such as age, gender, renal function, smoking and diabetes during follow-up for seven years (Sinkeler et al. 2012).

New drug molecules for the treatment of renal anemia that affect the HIF pathway and increase HIF concentration and thereby endogenous erythropoietin concentration have also had effects on patients'

cholesterol and glucose levels. In clinical trials, some effects have been positive, such as a decrease in serum total cholesterol level but a trend of increased glucose has also been noted (Haase 2017).

2.7 MARKERS OF IRON METABOLISM

2.7.1 Ferritin and serum transferrin receptor

Iron is an important cofactor involved in energy production, formation of hemoglobin and DNA synthesis and metabolism (Cairo et al. 2006). Iron intake, absorption, loss and storage determinate the total amount of iron in the body. Most of the iron is incorporated in erythrocytes in the form of hemoglobin, and 15 to 30 per cent is stored mainly in hepatocytes and reticuloendothelial macrophages. After absorption from the intestine, iron forms transferrin, which can be transported in plasma. In the cells iron combines with apoferritin and is stored in the form of ferritin (Andrews 1999). Transferrin receptors (TFR) are transmembrane proteins that mediate the cellular uptake of circulating iron (Kohgo et al. 2008). Transferrin receptors are also present in systemic circulation, and under iron-deficient states, the concentration of the cell surface as well as circulating TFRs are increased, reflecting cellular iron requirements (Kohgo et al., 2008). Ferritin is an indicator of iron stores in a healthy population and measurement of serum TFR has been accepted to serve as an index of tissue iron deficiency (Cook 2008, Kohgo et al. 2002, Zimmermann 2008).
Ferritin is a major iron storage protein that is responsible for the engagement and release of iron as well as regulation of iron availability (Zimmermann 2008). In iron deficiency, serum ferritin level decreases early before hemoglobin changes (Zimmermann 2008). However, ferritin is also an acute-phase protein and in inflammatory states a normal ferritin level does not exclude iron deficiency (Zandman-Goddard et al. 2007). Ferritin levels also increase in liver diseases and in hemochromatosis (Zandman-Goddard et al. 2007). Serum TFR levels do not rise under inflammatory conditions and can therefore serve as a marker of iron deficiency in patients with concomitant inflammation (Kohgo et al. 2008, Ferguson et al. 1992).

Serum ferritin levels rise when cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF) are present (Zandman-Goddard et al. 2007). Thus, serum ferritin levels rise in infections and inflammatory states (Zandman-Goddard et al. 2007). However, there is no evidence for regulated or active secretion of ferritin in humans in vivo (Kell et al. 2014). Actually, there is more evidence that serum ferritin originates from damaged cells and thus increased serum ferritin levels in inflammatory conditions reflect cellular damage (Kell et al. 2014). When leaking from damaged cells, ferritin loses most of its iron and leaving this iron in an unliganded form, can stimulate further cell damage (Kell 2010). The circumstances under which ferritin is normally degraded in vivo is not entirely understood and there exists little information of what happens to the iron content of the ferritin when the protein part of the ferritin molecule is degraded (Kell et al. 2014).

2.7.2 Iron metabolism markers and obesity

Previous studies have found higher serum ferritin levels in obese or overweight subjects compared to normal weight subjects and higher ferritin levels associated with visceral fat area compared to subcutaneous fat area (Ahmed et al. 2008, Iwasaki et al. 2005, Gillum et al. 2001). Obesity is associated with low-grade inflammation and infiltration of immune response-mediating cells, macrophages, in white adipose tissue (Bastard et al. 2006). Especially visceral white adipose tissue and infiltrated macrophages can serve as major sources of inflammatory cytokines, which are activators of ferritin transcription (Fahmhy et al. 1993). Adipose tissue can also modulate the systemic iron metabolism through the production of other adipokines, like adiponectin. An inverse relationship between serum adiponectin and ferritin levels has been found in cross-sectional and follow-up studies of subjects with prevalent or incident diabetes (Forouhi et al. 2007, Ku et al. 2009, Wlazlo et al. 2013).

However, although obese subjects exhibit high ferritin levels, epidemiological studies have found that obesity can be associated with iron deficiency as measured by circulating iron, transferrin saturation or transferrin receptor (TFR) levels (Zao et al. 2015). Studies suggest that TFR levels do not rise in inflammatory conditions and TFR could therefore serve as a marker of iron deficiency in patients with obesity (Ferjuson et al. 1992, Markovic et al. 2007).

Several mechanisms that affect iron metabolism could lead to iron deficiency and higher TFR levels seen in obesity. The iron-regulatory hormone hepcidin that is produced by the liver controls the dietary absorption, storage, and tissue distribution of iron. Adipose tissue expression of hepcidin has been shown to be enhanced in obese patients (Bekri et al. 2006). Hepcidin production is increased by inflammatory cytokines (Ganz 2011). High hepcidin levels are associated with reduced intestinal iron uptake and impaired release of iron from internal stores and thus could cause iron deficiency (Ganz 2007). In addition, hypoxia and the HIF pathway affect iron metabolism by many mechanisms. HIF increases the transcription of transferrin receptor (Tacchini et al. 1999).

2.7.3 Markers of iron metabolism, insulin resistance and type 2 diabetes

In addition to obesity, higher ferritin levels have been found to be associated with the risk of insulin resistance and incident type 2 diabetes in prospective studies (Forouhi et al. 2007, Frazier-Wood et al. 2011, Jehn et al. 2007, Kunutsor et al. 2013, Le et al. 2008, Montonen et al. 2012, Rajpathak et al. 2009, Salomaa et al. 2010, Zhao et al. 2012). High ferritin levels in insulin resistance and type 2 diabetes have previously been considered as a marker of iron overload although the impact of other mechanism like systemic inflammation could not have been ruled out (Bao et al. 2012, Kunutsor et al. 2013, Zhao et al. 2012). Iron overload theory has been supported by two studies that have found hepatic iron overload measured by magnetic resonance imaging in subjects with high ferritin concentration and insulin resistance and type 2 diabetes (Mendler et al. 1999, Zheng et al. 2011). The underlying mechanisms that could explain this mild iron overload contributing to insulin resistance and type 2 diabetes is unclear, although it is known from genetic overt iron overload diseases, such as hereditary hemochromatosis, that massive excessive accumulation of iron in tissues contributes to diabetes (Adams et al. 2005). It has been shown that elevated body iron stores impair glucose homeostasis by first increasing insulin resistance (Dandona et al. 1983).

It has been suggested that elevated body iron stores promote the development of insulin resistance by the peroxidation of lipids, especially free fatty acids, leading to accelerated production of free radicals, as iron is a pro-oxidant catalyst (Felber et al. 1987, Furukawa et al. 2004) The increase in free fatty acids oxidation causes decreased glucose uptake in the muscles, which stimulates gluconeogenesis in the liver and results in increased insulin resistance (Felber et al. 1987, Furukawa et al. 2004).

Also, higher ferritin levels have been associated with adipocyte insulin resistance, which is measured as the product of fasting insulin and free fatty acids concentrations. This suggests that iron metabolism may be involved in the development of insulin resistance not only in the liver and muscle but also in adipocytes (Wlazlo et al. 2013).

On the contrary, studies evaluating the relationship between serum transferrin receptor (TFR) and the risk of type 2 diabetes have been inconclusive. Higher TFR levels in type 2 diabetes patients or in subjects who developed type 2 diabetes during follow-up compared to control subjects have been found, reflecting tissue iron deficiency in developing type 2 diabetes (Fernandez-Cao et al. 2017, Joang et al. 2011, Rajpathak et al. 2009). However, also low TFR concentrations have been associated with an increased risk of developing type 2 diabetes or some studies have found no association between TFR and risk of type 2 diabetes (Aregbesola et al. 2013, Hernandez et al. 2005, Huth et al. 2015, Montonen et al. 2012). In a study of a population at a high risk of CVD, the association between TFR levels and risk of type 2 diabetes was dependent on the presence or absence of obesity and high waist circumference (Fernandez-Cao et al. 2017). Only in subjects with obesity and high waist circumference were elevated TFR levels associated with an increased risk of developing type 2 diabetes. Thus, abdominal obesity can alter the relationship between TFR and type 2 diabetes.

2.7.4 Iron metabolism markers, dyslipidemia and cardiovascular disease

Higher ferritin levels have been associated with dyslipidemia: high triglycerides and LDL cholesterol and low HDL cholesterol levels, including independently of hyperglycemia and insulin resistance (Li et al. 2017, Kim et al. 2016).

Conflicting results have been found between iron stores and the development of cardiovascular disease (Das de et al. 2015, Von Haehling et al. 2015). Previously, iron excess measured by serum ferritin levels was suggested to promote cardiovascular disease (Klipstein-Grobusck et al. 1999, Ma et al. 2002, Salonen et al.

1992, Valk et al. 1999). Iron has been suggested to induce the formation of reactive oxygen species and the peroxidation of lipids (Ma et al. 2002, Valk et al. 1999).

In more recent systematic reviews and meta-analysis no significant association has been found between serum ferritin and CVD (Das de et al. 2015, Von Haehling et al. 2015), instead it has even been concluded that high body iron stores could confer protection against the development of CVD. This was found also in a prospective study where low iron status measured by serum ferritin was associated with a higher cardiovascular disease incidence in patients with type 2 diabetes (Suarez-Ortegon et al. 2018). In a study of type 2 diabetes patients with coronary artery disease (CAD) high levels of TFR and both low and high levels of serum ferritin identified patients who had a poorer prognosis (Ponikowska et al. 2013). Higher TFR levels and the lowest and the highest quintiles of ferritin were associated with cardiovascular hospitalization and mortality independently of hemoglobin and markers of inflammation (Ponikowska et al. 2013).

2.8 LIPOPROTEIN PARTICLES

2.8.1 Assessment of lipoprotein particles

Lipids are transported in circulation in the form of lipoprotein particles, which have a core consisting of cholesteryl esters and triglycerides and a surface of free cholesterol, apolipoproteins and phospholipids. Lipoprotein particles have various densities, sizes, compositions and functions and they can be classified based on particle size, electrophoretic mobility, apolipoprotein content or hydrated density in ultracentrifugation (UC) (Dominiczak et al. 2000, Havel et al. 1955, Noble et al. 1969,). The major lipoprotein classes: chylomicrons, very-low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are defined according to their densities using UC (Gotto et al. 1986).

Chylomicrons that are derived from the intestine constitute together with liver-secreted VLDL particles the triglyceride-rich lipoproteins. In circulation, lipoprotein lipase hydrolyzes VLDL particles` triglycerides and releases fatty acids, thus generating small triglyceride-depleted lipoproteins: IDL ja LDL particles. The excess in VLDL particles production that is seen in insulin resistance, hyperglycemia and metabolic syndrome (Adiels et al. 2008) can result in increased amounts of triglycerides transferred to LDL particles. These triglyceride-rich LDL particles can further be hydrolyzed by hepatic lipase, producing smaller and denser LDL particles (Tan et al. 1995). HDL particles can originate from the liver and intestine or they can be synthesized from remnant surface components of chylomicrons and VLDL particles (Eisenberg 1984).

All lipoprotein particles are heterogenous in size and composition. A proton nuclear magnetic resonance (NMR) technique for lipoprotein quantification using a spectroscopic method was first reported in 1991 (Otvos et al. 1991). Proteins submitted to a high-frequency magnetic field produce resonance spectra that are specific to their chemical environment (Otvos et al. 1991). Lipoproteins in plasma have specific resonance signatures and a relationship between NMR-measured resonance frequency and lipoprotein size or diameter has been demonstrated (Lounila et al. 1994, Otvos 2000).

Lipoprotein NMR spectroscopy measures the specific resonance signature of the particles' methyl groups. Different lipoproteins have different lipid signals depending on their size. Also, the amplitude of the lipid resonance reflects the amount of lipids in the particle. (Jeyarajah et al. 2006). Thus, NMR analysis produces specific signals and amplitudes of lipoproteins which are the lipoproteins` diameter and concentration. The development of automatic assays using the NMR method has enabled its more widespread use in clinical trials and its clinical relevance for cardiovascular disease risk assessment has been demonstrated (Clouet-Foraison et al. 2017, Cole et al. 2013, Mora et al. 2010).

2.8.2 Lipoprotein particles and obesity, metabolic syndrome and type 2 diabetes

Changes in NMR-measured lipoprotein particles concentrations and sizes have been demonstrated in obesity, metabolic syndrome, impaired glucose tolerance and type 2 diabetes.

MetS and all its individual components are characterized by a reduction in LDL and HDL particle sizes (Frasier-Wood et al. 2011, Kathiresan et al. 2006). Larger VLDL particles have been associated especially

with high glucose or diabetes, triglycerides and the waist circumference components of MetS (Frasier-Wood et al. 2011).

Increasing body weight has been associated most clearly with increasing levels of large VLDL, and to a less extend with increasing small LDL particles concentrations. Weight loss has been associated with a decreasing size of VLDL particles and an increased number of large HDL particles (Nagamuna et al, 2009, Mäntyselkä et al. 2012).

In cross-sectional studies, individuals with impaired fasting glucose or glucose tolerance have had increased concentrations of VLDL subclass particles, especially larger VLDL particles, and decreased concentrations of larger HDL particles (Wang et al. 2012). In prospective studies, increased concentrations of large HDL particles have shown to be preventive for hyperglycemia and incident type 2 diabetes (Abbasi et al. 2013, Fagot-Champagna et al. 1999, Fizelova et al. 2015,). On the contrary, increased levels of small HDL, small LDL and large VLDL particles have been shown to be associated with an increased risk of developing hyperglycemia and type 2 diabetes (Austien et al. 1995, Fagot-Champagna et al. 1999, Fizelova et al. 2015, Mora et al. 2010)

2.8.3 Lipoprotein particles and cardiovascular disease

It has been suggested that measuring the size and concentration of lipoprotein particles could improve cardiovascular disease risk evaluation when added to standard measurements of HDL and LDL cholesterol and triglycerides (Krauss 2010, Jellinger et al. 2012).

However, the association between changes in lipoprotein particles sizes or concentrations and CVD risk have been more complicated to evaluate than that of lipoprotein particles and hyperglycemia or diabetes, thus the evidence from prospective studies is so far fairly discordant (Krauss 2010). The strongest associations with cardiovascular risk have been found with lower concentrations of large HDL particles or with smaller HDL particle size (El Harchaoui et al. 2009, Mutharasan et al. 2017, Würtz et al. 2015) and with a decrease in large LDL particles by a corresponding increase in small LDL particles number (Pichler et al., 2018, Williams et al., 2014).

3 AIMS OF THE STUDY

The general aims of this study were to investigate whether the biomarkers of adipose tissue dysfunction and hypoxia as well as of iron metabolism are associated with prevalent as well as incident metabolic syndrome and its components and to test whether an atherogenic lipoprotein particle profile is associated with hemoglobin level.

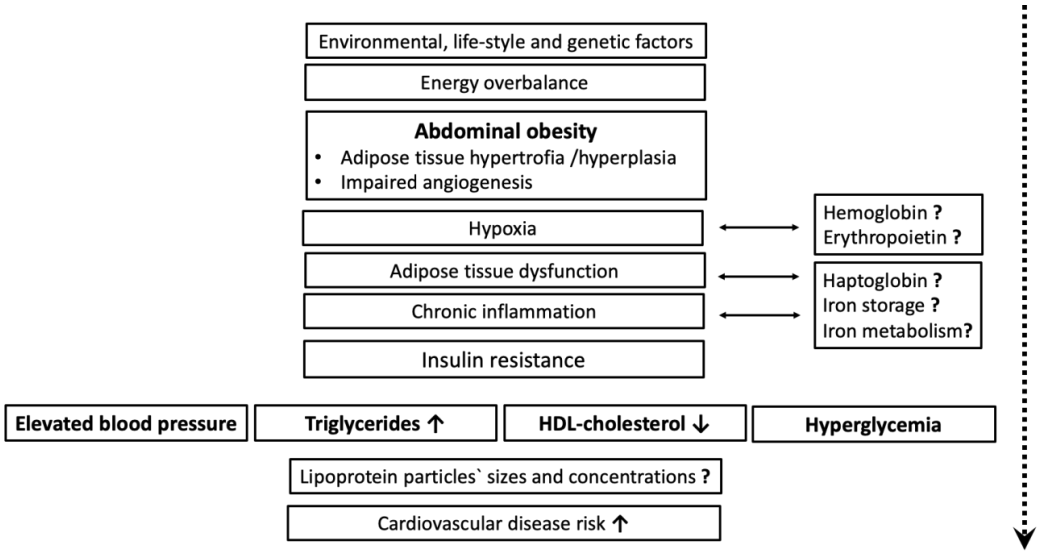

Figure 1. Pathophysiological factors influencing the development of Mets and cardiovascular disease risk. Unknown factors (**?**) marking the research questions of this thesis.

The more detailed research questions were:

I. Is erythropoietin, hemoglobin, haptoglobin, ferritin and transferrin receptor level associated with prevalent metabolic syndrome (MetS) and individual MetS components?
II. Is ferritin level associated with incident MetS and its components?
III. Are lipoproteins VLDL, LDL and HDL particles` sizes and concentrations associated with hemoglobin level?

4 SUBJECTS AND METHODS

4.1 STUDY POPULATION AND DESING

The analyses of the present study are based on the baseline and 6.5-year follow-up data of initially 1,294 middle-aged subjects who lived in the town of Pieksämäki, Finland, and were born in 1942, 1947, 1952, or 1962. The subjects were picked from population register data updated in February 1992 without any excluding criteria.

Written invitations were mailed according to the civil register and repeated two separate times to invite these subjects to a health care visit in the years 1997–1998 initially, and to a follow-up visit in 2003–2004. A total of 923 (71%) participated in the first health care visit and 766 (59%) subjects in the second visit.

In study I, the final analysis included data from 766 (425 women and 341 men) subjects who participated in a second health care visit in 2003–2004.

In study II, analysis included data from 923 subjects who participated in the first health care visit in 1997-1998 (baseline), and 693 subjects who attended both baseline and control 2003-2004 visits. All variables finally analyzed in study II were available from 691 subjects (289 men and 402 women).

In study III, the final analysis included data from 766 subjects (425 women and 341 men) who participated in a second health care visit in 2003–2004 (Figure 2).

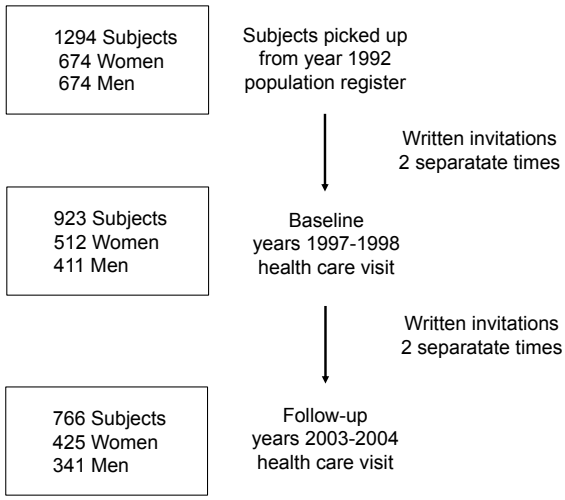

Figure 2. Study population and design.

4.2 METHODS

4.2.1 Clinical methods

All subjects filled in a questionnaire about their medical history and their current medical condition. Subjects were interviewed and examined by two nurses who were specially trained for this task by a researcher physician in 1997.

Subjects were asked about their smoking habits, alcohol consumption, and physical activity. Subjects who smoked daily were considered to be current smokers. Alcohol consumption was divided into three categories: low, meaning no alcohol use; moderate (less than two portions = 10-14 grams of alcohol per day); and high (more than two portions of alcohol per day). Physical activity was considered to be high in

subjects who exercised daily for at least 30 minutes in their leisure time, moderate in subjects who exercised at least three times per week, and low if exercising frequency was less than three times per week.

Two nurses performed the study processing and physical examination. Weight while wearing light clothing and height was measured to an accuracy of 0.1 kg and 0.5 cm, respectively. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured with a mercury sphygmomanometer in a sitting position after 15 minutes of rest. The measurement was repeated after five minutes. The mean of the two measurements was used in the statistical analyses. Waist circumference was measured from the midpoint between the lateral iliac crest and the lowest rib to an accuracy of 0.5 cm.

4.2.2 Biochemical methods

Fresh blood samples were drawn after an overnight (12 hours) fast. Plasma was separated by centrifugation for the determination of glucose and lipids and the samples were frozen immediately and stored at -70 °C until they were analyzed in the scientific laboratory of Kuopio University Hospital during years 2009 and 2010.

Plasma glucose concentration was measured by an automated colorimetric method (Peridochrom Glucose GOD- PAP, Boehringer, Germany). Triglycerides were measured by enzymatic colorimetric methods (CHOD-PAP, GPO-PAP, Boehringer Mannheim GmbH, Germany). HDL cholesterol was measured by the same method after precipitation of low-density lipoprotein cholesterol and very low-density lipoprotein cholesterol with phosphotungestic acid and magnesium.

High-sensitivity C-reactive protein (hs-CRP) was measured with an Immulite analyzer and a DPC high-sensitivity CRP assay (DPL, Los Angeles, CA, USA).

White blood cell and platelet count, hemoglobin and hematocrit were measured using an automatic electronic cell calculator. Erythropoietin (EPO) concentration was analyzed using an immunoluminometric assay method. Soluble transferrin receptor concentration (TFR) was measured by a particle enhanced immunoturbidimetric assay (Cobas c systems, Roche Diagnostics GmbH, Mannheim, Germany). Ferritin concentration was analyzed using an electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). The analytical method for haptoglobin concentration measurements was an immunoturbidimetric assay (Cobas c systems, Roche Diagnostics GmbH, Mannheim, Germany, ACN 228). Creatinine was measured using an enzymatic method.

In study III, the homeostasis model for assessment of insulin resistance (HOMA-IR) was used in the second adjusted model. The HOMA-IR was calculated with the following formula: HOMA-IR = fasting plasma glucose (mmol/L) × fasting plasma insulin (μU/ml) / 22.5 (Matthews et al., 1985). Plasma insulin was determined using the Phadesph Insulin radioimmunoassay (RIA) 100 method (Pharmacia Diagnostics AB, Uppsala, Sweden) in the scientific laboratory of Kuopio University Hospital.

In study III, concentrations and sizes of lipoprotein subclass particles were analyzed with high-throughput NMR spectroscopy of native serum samples in 2009. NMR data were measured at 37 °C using a Bruker AVANCE III spectrometer operating at 500.36 MHz using a new automated platform (Vehtari et al. 2007, Soininen et al. 2009). The following 14 lipoprotein subclasses were calibrated using high-performance liquid chromatography: chylomicrons (CMs) and the largest VLDL particles (CM/ largest VLDL; average particle diameter ± 75 nm); five different VLDL subclasses, i.e., very large (average particle diameter 64.0 nm), large (53.6 nm), medium (44.5 nm), small (36.8 nm) and very small VLDL (31.3 nm); intermediate density lipoprotein (IDL; 28.6 nm); three LDL subclasses, i.e., large (25.5 nm), medium (23.0 nm), and small LDL (18.7 nm); and four HDL subclasses, i.e., very large (14.3 nm), large (12.1 nm), medium (10.9 nm), and small HDL (8.7 nm). In the analyses, VLDL particles included all sizes of VLDL particles including the largest VLDL particles and chylomicrons. LDL particles included IDL particles and all sizes of LDL particles. HDL particles included all sizes of HDL particles.

4.2.3 Statistical methods

In study I and II the results are expressed as means and standard deviations (SDs) for continuous variables and as proportions for categorical variables. The normality of variables was evaluated by the Shapiro-Wilk test. Statistical comparisons between the groups were performed using the chi-square test, t-test, or bootstrap-type t-test as appropriate. Bootstrap type analysis of covariance was also used to compare the

groups as measurements. In these analyses in study I, age values, sex, smoking, physical activity and hs-CRP values were used as covariates.

In study II, the baseline variables of age, smoking, physical activity, serum ferritin levels, body mass index and hs-CRP were used as covariates. Partial correlations were calculated between a change in serum ferritin level and changes in levels of MetS components and adjusted for age, and baseline smoking, physical activity, alcohol use, serum ferritin concentration, body mass index, and hs-CRP.

In study III, the data are presented as means and standard deviations. The 95% confidence intervals for the lipoprotein particle concentrations and diameters were obtained by bias-corrected, accelerated bootstrapping. Associations between the serum triglyceride, HDL, and total cholesterol concentrations with the NMR-measured concentrations were estimated with regression analysis using Sidak-adjusted probabilities. Multiple linear regression analysis was used to estimate the independent impacts of LDL, HDL, and VLDL particle diameter on the hemoglobin stratified by sex.

The analyses were carried out with SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. IBM Corp) and Stata 14.0, StataCorp LP (College Station, TX, USA).

In studies I, II and III, in all hypotheses, $p < 0.05$ was considered significant.

4.3 DETERMINATION OF THE METABOLIC SYNDROME

In studies I ja II, MetS was defined according to the new harmonized criteria (Alberti et al., 2009). Subjects with three or more of the following components were classified as having MetS: (1) increased waist circumference (≥102 cm (≥40 in) for men and ≥88 cm (≥35 in) for women); (2) elevated fasting total triglycerides (≥1.7 mmol/l (≥150 mg/dl) or treatment for dyslipidemia); (3) low fasting serum high density lipoprotein (HDL) cholesterol (<1.03 mmol/l (<40 mg/dl) in men or <1.29 mmol/l (<50 mg/dl) in women or treatment for dyslipidemia); (4) systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or the use of antihypertensive medication; and (5) fasting plasma glucose ≥5.6 mmol/l (≥100 mg/dl) or the use of antihyperglycemic medication. Subjects having treatment for dyslipidemia were classified as having the HDL and triglyceride components of MetS.

4.4 ETHICAL CONSIDERATION

All the subjects were informed about the aims and methods of the study by the invitation letters and in the first interview. All the participants gave written consent before participation. The study protocol was approved by the Ethics Committee of Kuopio University Hospital and the University of Kuopio.

5 RESULTS

5.1 CHARACTERISTICS OF THE STUDY POPULATION

At baseline in 1997-1998, MetS was present in 31% of the study population. During the 6.5-year follow-up time until 2003-2004, 122 (18%) incident cases of MetS developed and 44 (6%) cases of MetS resolved. In 2003-2004 MetS was present in 52% of women and in 48% of men. Both women and men with MetS were significantly older than the subjects without. Current smoking did not differ significantly between subjects with or without MetS. In those with MetS, 14% of female and 26% of male subjects were classified as current smokers. In those without MetS, the proportions of female and male smokers to non-smokers were 19% and 25%, respectively. Use of alcohol did not differ significantly in male subjects with or without MetS. Female subjects without MetS currently used more alcohol compared to female subjects with MetS. Blood pressure was the most common component of MetS. It was present in 78% of the men and 66% of the women. High fasting plasma glucose was also present in a large part of the subjects (75% of the men and 53% of the women).

Among the population of the follow-up visit, 681 were the same subjects as in the baseline visit (74%). In study II more of the 242 subjects of the 923 participants studied in 1996–1997 who did not participate in the second health care visit in 2003–2004 lived alone (30% of the nonparticipants vs. 21% of the participants; $P = 0.045$), smoked (45% vs. 28%; $P < 0.001$), and used on average at least 2 units/day of alcohol (13% vs. 6%; $P = 0.003$). Biochemical measurements, prevalence of the metabolic syndrome, and use of medication for hypertension, diabetes, and dyslipidemia were similar in both groups. In the baseline, use of medication for dyslipidemia as well as for diabetes was uncommon (n=16, 2%; n=7, 1%, respectively). At the follow-up, use of medication had increased: 119 subjects (17%) for dyslipidemia and 39 (6%) for diabetes. The clinical and life-style characteristics of the study population are presented in Table 3.

Table 3. Clinical and life-style characteristics of the study population in 2003-2004.

Characteristics	Male N = 341		p	Female N = 425		p
	MetS present N = 159	MetS not present N = 182		MetS present N = 170	MetS not present N = 255	
Age, years **	53.7 (5.8)	50.7 (6.2)	<0.001	54.4 (5.7)	50.5 (6.4)	<0.001
Body mass index, kg/m^2 **	29.8 (3.9)	25.2 (2.5)	<0.001	30.8 (5.3)	25.1 (3.5)	<0.001
Waist, cm **	103.8(10.7)	89.6 (7.2)	<0.001	96.2 (12.4)	81.0 (8.5)	<0.001
FP-gluc mmol/L **	6.5 (1.4)	5.8 (0.8)	<0.001	6.3 (1.5)	5.5 (0.4)	<0.001
BP systolic, mmHg **	143 (19)	136 (17)	<0.001	144 (17)	131 (16)	<0.001
BP diastolic, mmHg **	87 (9)	82 (10)	<0.001	86 (9)	79 (8)	<0.001
HDL-C, mmol/L **	1.3 (0.4)	1.6 (0.4)	<0.001	1.6 (0.4)	1.8 (0.3)	<0.001
Trigly, mmol/L **	1.9 (1.5)	1.1 (0.5)	<0.001	1.6 (0.8)	1.0 (0.3)	<0.001
Creatinine µmol/L **	88.5 (11.9)	85.9 (8.7)	0.02	75.3 (14.8)	73.8 (8.9)	0.17
Hemoglobin (g/L) **	154 (9)	150 (9)	<0.001*	141 (10)	136 (9)	<0.001*
Erythropoietin (I/U) **	10.7 (6.5)	9.0 (3.3)	<0.01*	12.6 (10.0)	10.8 (6.7)	0.04*
Alat (I/U) **	38 (17)	28 (11)	<0.01	31 (15)	23 (12)	< 0.01
Ferritin (µg/L) **	216 (165)	151 (112)	<0.001*	94 (75)	61 (48)	<0.001*
TFR (mg/L) **	2.9 (2.8)	2.6 (0.6)	0.12*	2.8 (0.9)	2.7 (1.0)	0.32*
Haptoglobin (g/L) **	1.3 (0.6)	1.1 (0.5)	0.012*	1.4 (0.6)	1.2 (0.4)	<0.001*
Hs-CRP (mg/L) **	2.4 (3.5)	1.5 (2.9)	0.058*	3.1 (3.4)	1.5 (2.3)	<0.001*
Components of MetS:						
Waist n (%)	102 (64)	8 (4)	<0.001	134 (79)	40 (16)	<0.001
FP-glucose n (%)	144 (91)	113 (62)	<0.001	139 (83)	85 (33)	<0.001
Blood pressure n (%)	146 (92)	119 (65)	<0.001	154 (90)	126 (49)	<0.001
HDL-cholesterol n (%)	90 (57)	2 (1)	<0.001	101 (59)	10 (4)	<0.001
Triglycerides n (%)	116 (73)	16 (9)	<0.001	118 (69)	6 (2)	<0.001
Life-style factors, n (%):						
Current smoker	42 (26)	46 (25)	0.81	25 (14)	49 (19)	0.23
Current use of alcohol			0.055			0.038
Low (nothing)	20 (12)	26 (14)		46 (28)	46 (18)	
Moderate	68 (43)	97 (54)		97 (58)	177 (70)	
High	71 (45)	58 (32)		24 (14)	31 (12)	
Physical activity n (%)			0.11			0.83
Low	33 (21)	56 (31)		55 (33)	80 (32)	
Moderate	97 (61)	96 (53)		92 (55)	146 (57)	
High	29 (18)	30 (16)		21 (12)	28 (11)	

*Values are adjusted for age, ** Mean (SD) Abbreviations: Fp-gluc, fasting plasma glucose; BP systolic/diastolic, systolic/diastolic blood pressure; HDL-C, high density cholesterol; Trigly, triglycerides; ALAT, alanine aminotransferase; TFR, transferrin receptor; Hs-CRP, high sensitivity c-reactive protein.

5.2 ERYTHROPOIETIN, HEMOGLOBIN, HAPTOGLOBIN, FERRITIN, AND TRANSFERRIN RECEPTOR IN METABOLIC SYNDROME (STUDY I)

Mean erythropoietin level was significantly higher in subjects with MetS than subjects without even after adjusting for sex ($p = 0.018$, Figure 3A). Mean ferritin and mean hemoglobin were significantly higher in subjects with MetS than without ($p < 0.001$, Figures 3B, C). Mean haptoglobin was significantly higher in subjects with MetS ($p = 0.018$, Figure 3D). Mean TFR did not differ significantly between subjects with or without MetS (Figure 3E). Mean hemoglobin was significantly higher in subjects with any of the MetS components (abdominal obesity, blood pressure (BP), low HDL, high triglycerides (TG) or elevated glucose, Figure 3C).

Mean ferritin was significantly higher in subjects with abdominal obesity or low HDL or high TG or elevated glucose component (Figure 3B). Mean erythropoietin was significantly higher in subjects with abdominal obesity component but did not differ significantly between subjects with or without other components of MetS (Figure 3A). Mean haptoglobin was significantly higher in subjects with the blood pressure or elevated glucose component (Figure 3D). Mean TFR was significantly higher in subjects with the abdominal obesity component but did not differ significantly between subjects with or without other components of MetS (Figure 3E).

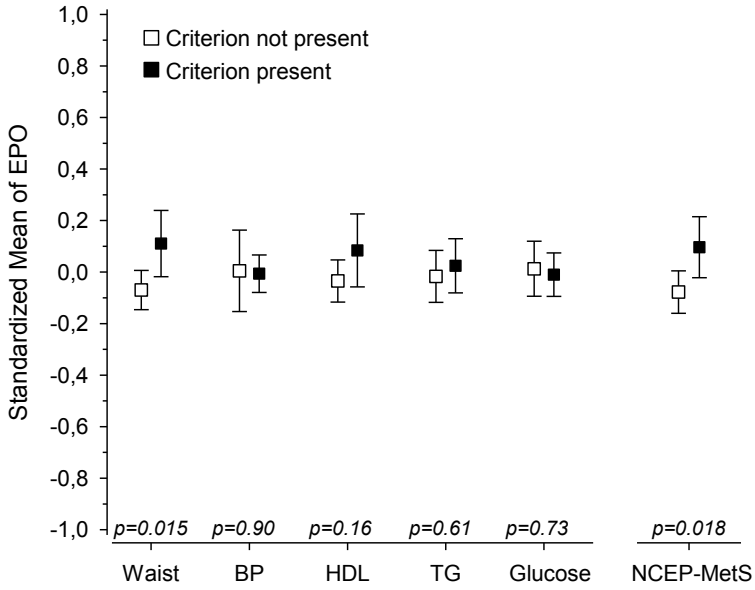

Figure 3 A. Erythropoietin mean values and individual MetS components.

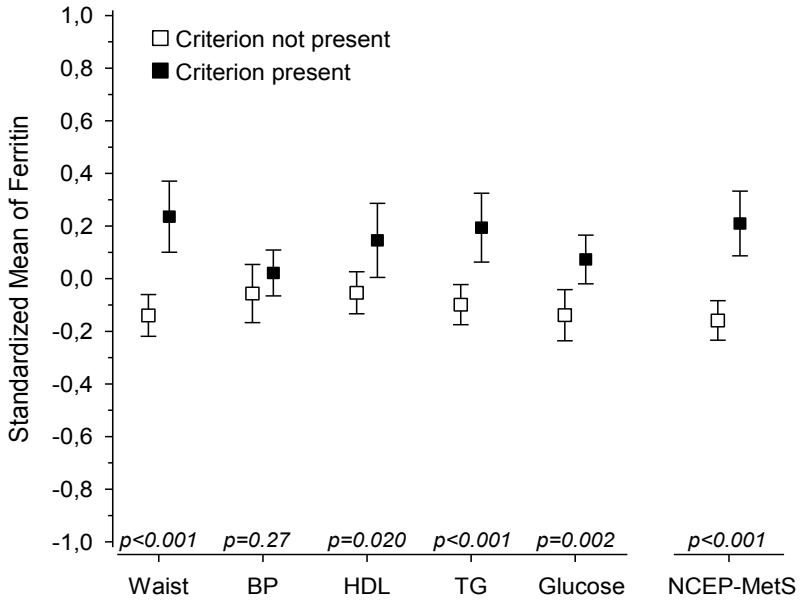

Figure 3 B. Ferritin mean values and individual MetS components.

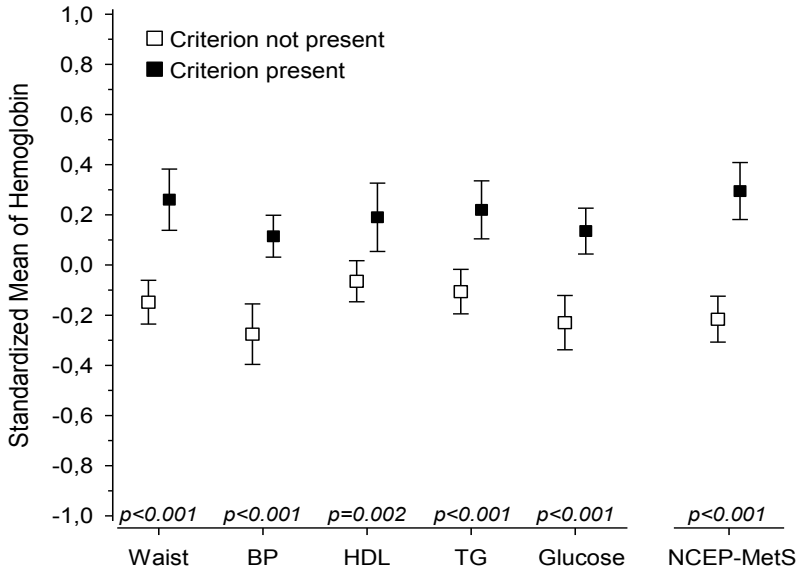

Figure 3 C. Hemoglobin mean values and individual MetS components.

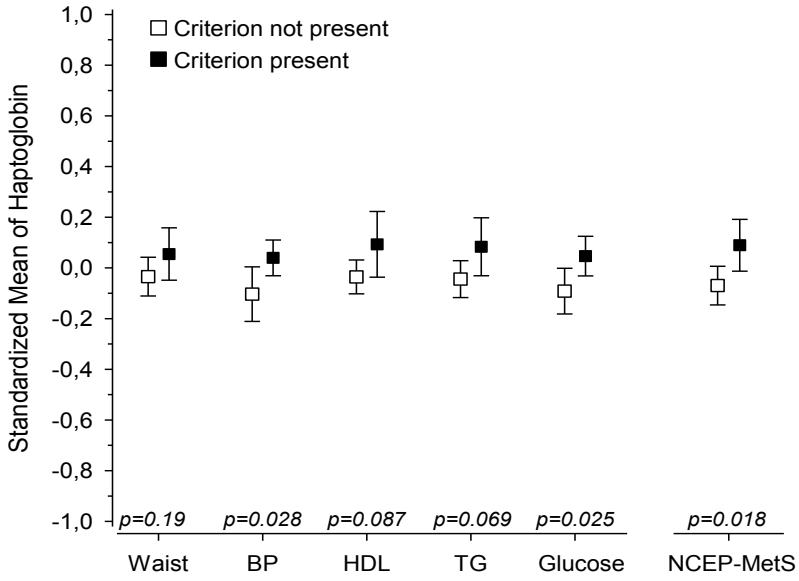

Figure 3 D. Haptoglobin mean values and individual MetS components.

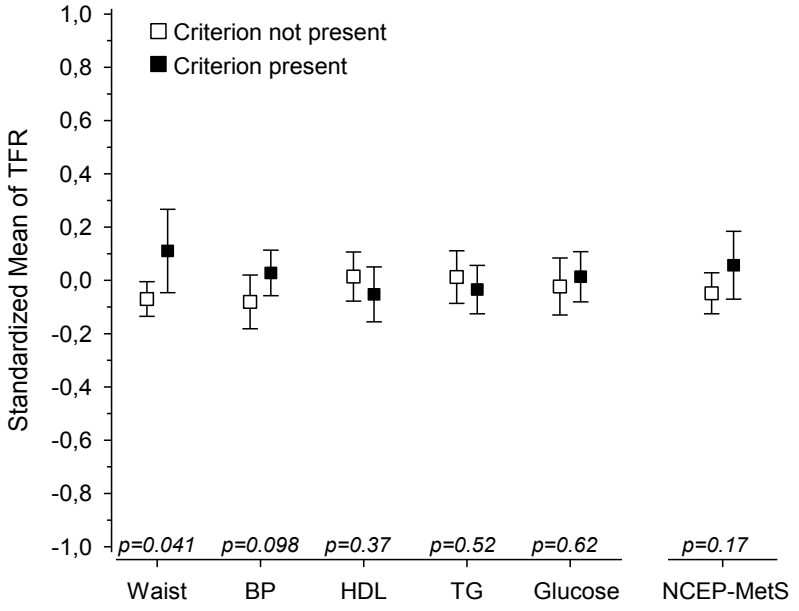

Figure 3 E. Transferrin receptor mean values and individual MetS components.

All values are standardized for age, sex, hs-CRP, smoking and physical activity. Abbreviations: BP, blood pressure; EPO, erythropoietin; HDL, high density lipoprotein cholesterol; TFR, transferrin receptor; TG, triglycerides. NCEP (National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults) criteria of MetS: Waist >102 cm(male) or >88 cm(female); FP-glucose ≥ 5.6 mmol/L; Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication; HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia; triglycerides >1.7 mmol/l or medication for dyslipidemia.

5.3 CHANGE IN FERRITIN LEVEL AND METABOLIC SYNDROME DURING A 6.5 YEAR FOLLOW-UP (STUDY II)

5.3.1 Ferritin level changes and development or resolution of MetS and its components

Serum ferritin level increased significantly more both in women and men who developed MetS criteria during the 6.5 years compared with women and men who did not develop MetS (p = 0.04, p = 0.03, respectively). Serum ferritin levels increased significantly less in women in whom the criteria for MetS resolved during the 6.5-year period compared with women in whom the MetS criteria remained (p = 0.01, Figure 4A).

Significant changes in relation to resolving or developing the criteria of MetS were found in the glucose, triglyceride and waist components of MetS. Increases in serum ferritin levels were significantly lower in women in whom the glucose criterion of MetS resolved during the follow-up period compared with women in whom the glucose criterion remained (p = 0.01, Figure 4B). Also, Serum ferritin levels decreased or increased significantly more in men in whom the MetS criteria for triglycerides resolved or developed during the follow-up period (p = 0.004, p = 0.05, respectively, Figure 4C). Additionally, the increase in serum ferritin level was significantly higher in women who developed the waist criterion during the follow-up period (p = 0.0001, Figure 4D). Changes in ferritin levels between subjects whose HDL or blood pressure criterion developed or resolved during the follow-up time did not differ significantly Figure 4E, F).

All results are adjusted for age, baseline smoking, baseline physical activity, baseline use of alcohol, baseline serum ferritin level, baseline body mass index and baseline hs-CRP.

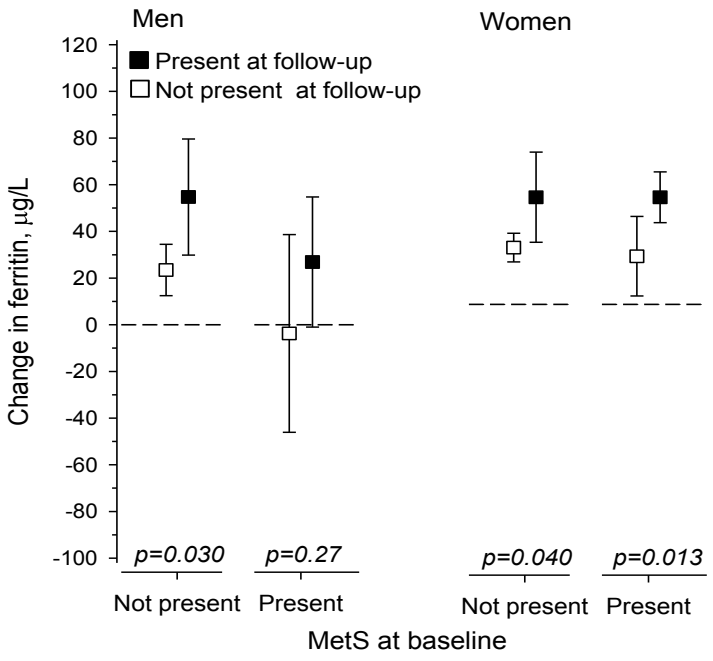

Figure 4 A. Serum ferritin level changes and development or resolving of MetS during the 6.5-year follow-up.

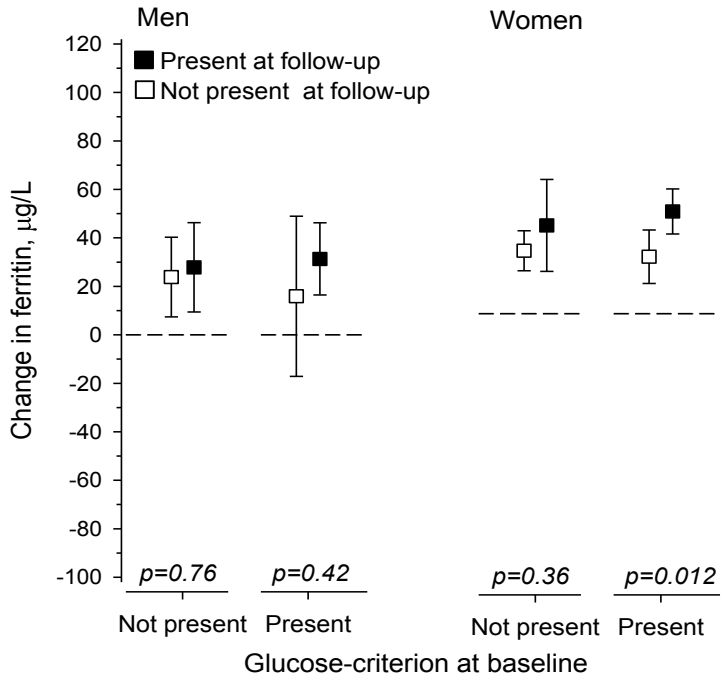

Figure 4 B. Serum ferritin level changes and development or resolving of MetS glucose-criterion during the 6.5-year follow-up.

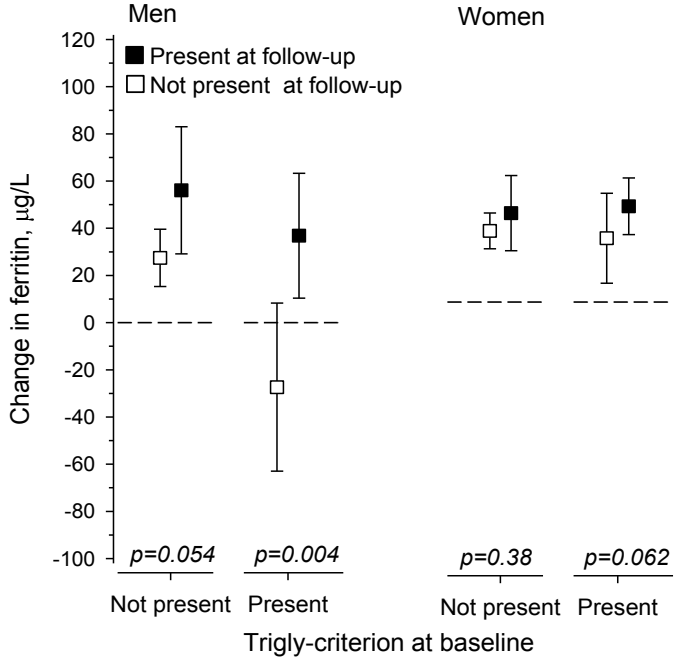

Figure 4 C. Serum ferritin level changes and development or resolving of MetS triglyceride-criterion during the 6.5-year follow-up

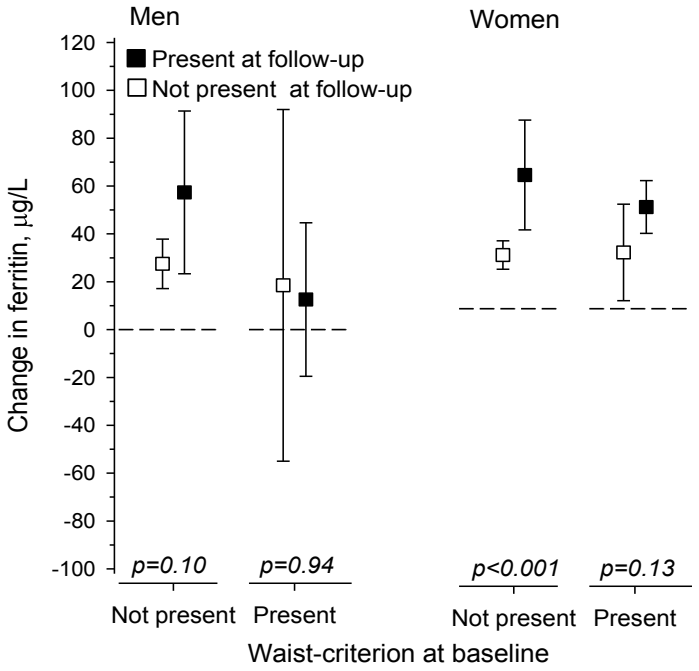

Figure 4 D. Serum ferritin level changes and development or resolving of MetS waist-criterion during the 6.5-year follow-up.

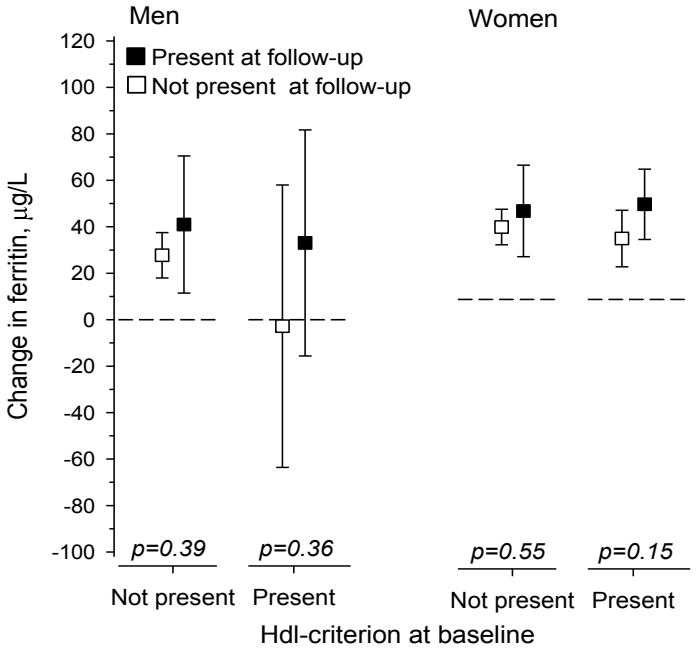

Figure 4 E. Serum ferritin level changes and development or resolving of MetS high density lipoprotein -criterion during the 6.5-year follow-up.

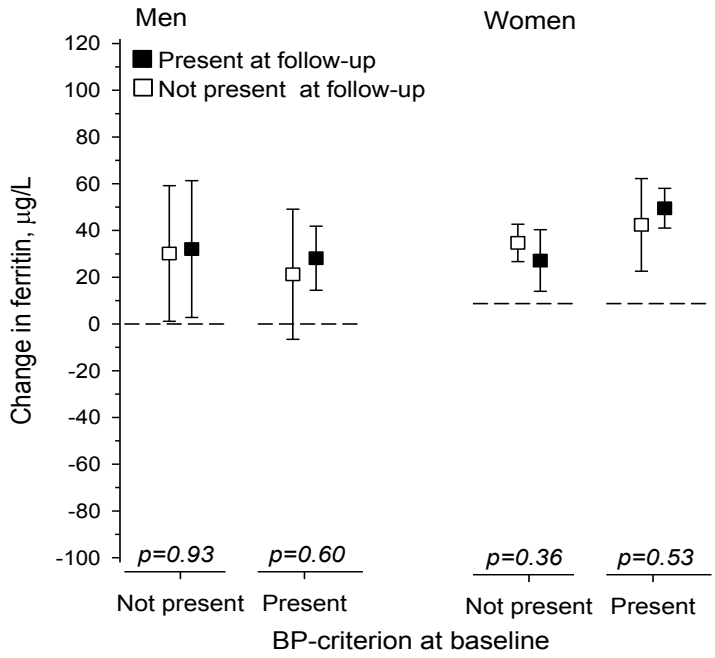

Figure 4 F. Serum ferritin level changes and development or resolving of MetS blood pressure -criterion during the 6.5-year follow-up. All results are adjusted for age, baseline smoking, baseline physical activity, baseline use of alcohol, baseline serum ferritin level, baseline body mass index and baseline hs-CRP.

5.3.2 Association between change in ferritin level and MetS components

There was a significant positive correlation between change in serum ferritin level and change in waist circumference both in men and women ($p < 0.001$, $p < 0.01$, respectively). In addition, positive correlations between change in serum ferritin level and change in plasma triglyceride as well as glucose levels were significant in men ($p < 0.001$). There was a significant negative correlation between change in serum ferritin and change in plasma HDL cholesterol level both in men and women. The correlations between change in serum ferritin level and change in systolic or diastolic blood pressure level were not significant (Table 4).

Table 4. Correlations between change in serum ferritin level and change in Mets components.

MetS Components	Men r (95% CI)	Women r (95% CI)
Waist	0.21 (0.11 to 0.35) ***	0.15 (0.05 to 0.24) **
Triglycerides	0.20 (0.11 to 0.31) ***	0.09 (-0.01 to 0.17)
HDL-C	-0.12 (-0.25 to -0.01) *	-0.13 (-0.22 to -0.02) *
Glucose	0.20 (0.07 to 0.33) ***	0.08 (-0.02 to 0.18)
Systolic BP	-0.03 (-0.15 to 0.11)	0.02 (-0.07 to 0.12)
Diastolic BP	0.03 (-0.11 to 0.18)	0.03 (-0.09 to 0.13)

* p < 0.05, ** p < 0.01, *** p < 0.001
Adjusted for age, baseline smoking, baseline physical activity, baseline use of alcohol, baseline serum ferritin level, baseline body mass index and baseline high sensitivity C-reactive protein.
HDL: high density cholesterol; Systolic BP: systolic blood pressure; Diastolic BP: diastolic blood pressure.

5.4 LIPOPROTEIN PARTICLE SIZE, CONCENTRATION AND HEMOGLOBIN (STUDY III)

5.4.1 Correlations between plasma triglycerides, HDL or total cholesterol and NMR-measured LDL, HDL or VLDL particle concentration

Total plasma cholesterol had a high positive correlation with NMR-measured LDL particle concentration in both women and men ($p < 0.001$). Also, plasma total cholesterol had a moderate positive correlation with NMR-measured VLDL concentration in women and men ($p < 0.001$). Plasma triglycerides had a high positive correlation with the NMR-measured VLDL particle concentration in women ($p < 0.001$) and a moderate positive correlation in men ($p < 0.001$). Plasma HDL cholesterol had a high positive correlation with the NMR-measured HDL particle concentration in both women and men ($p < 0.001$). (Table 5) There was no significant correlation between plasma triglycerides, HDL, or total cholesterol and NMR-measured LDL, HDL, or VLDL particle diameter.

Table 5. Correlations between plasma triglycerides, HDL or total cholesterol and NMR-measured LDL, HDL or VLDL particle concentration.

Plasma cholesterol	**NMR-measured particle concentrations**		
	LDL r	**HDL r**	**VLDL r**
Women			
Triglycerides	0.29[a]	-0.07	0.85[a]
HDL	0.09	0.67[a]	-0.37[a]
Total	0.92[a]	0.25[a]	0.50[a]
Men			
Triglycerides	-0.01	-0.03	0.58[a]
HDL	0.16[b]	0.70[a]	-0.45[a]
Total	0.89[a]	0.34[a]	0.43[a]

[a] $p < 0.001$, [b] $p < 0.05$; Sidak-adjusted probabilities.

5.4.2 LDL, HDL and VLDL particle diameter change in relation to hemoglobin concentration

Larger VLDL particle diameter was associated with higher hemoglobin concentrations in both men and women ($p = 0.002$ and $p = 0.029$, respectively). There was a strong relationship between smaller HDL particle size and higher hemoglobin concentration in both men and women as well as lower LDL particle size and higher hemoglobin concentration in men ($p < 0.001$). Also, lower LDL particle size was associated with higher hemoglobin concentrations in women ($p = 0.002$). These results were adjusted for age, hs-CRP and LDL, HDL or VLDL NMR-measured particle concentration (Figure 5 A).

An adjusted model was added to the homeostasis model for assessment of insulin resistance (HOMA-IR) (Figure 5 B). After adjusting for HOMA-IR all results remain significant except for larger VLDL diameter in women ($p = 0.073$).

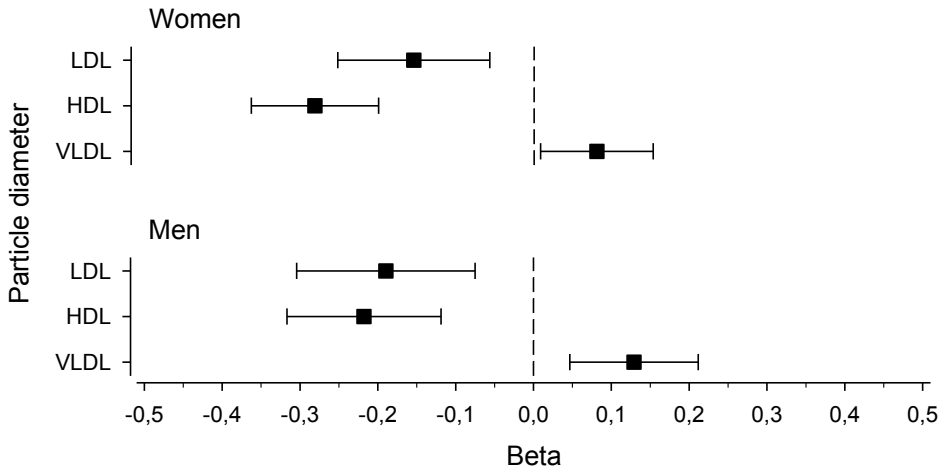

Figure 5 A. LDL, HDL and VLDL particle diameter changes in relation to hemoglobin concentration in women and men.
Beta = Hemoglobin standardized coefficients
Women: LDL particle size p=0.002, HDL particle size p<0.001, VLDL particle size p=0.029
Men: LDL particle size p<0.001, HDL particle size p<0.001, VLDL particle size p=0.002
All values are adjusted for age, hs-CRP and LDL, HDL or VLDL NMR-measured particle concentration.

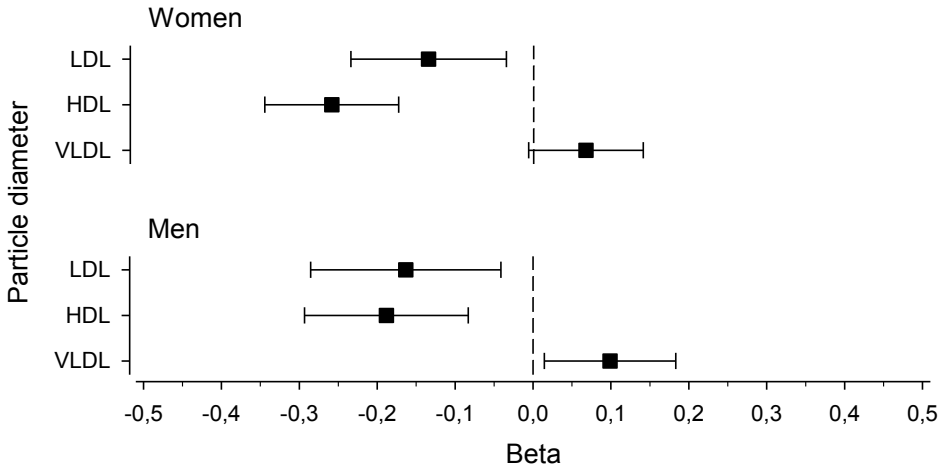

Figure 5 B. LDL, HDL and VLDL particle diameter changes in relation to hemoglobin concentration in women and men.
Beta = Hemoglobin standardized coefficients
Women: LDL particle size p=0.008, HDL particle size p<0.001, VLDL particle size p=0.073
Men: LDL particle size p=0.009, HDL particle size p<0.001, VLDL particle size p=0.003
All values are adjusted for age, hs-CRP, and HOMA-IR, and LDL, HDL or VLDL NMR-measured particle concentration.

5.4.3 Correlations between hemoglobin and NMR-measured VLDL, LDL, and HDL particle concentration

VLDL particle concentration had a positive correlation with hemoglobin concentration ($r = 0.15$; $p < 0.001$, Figure 6 A). LDL particle concentration showed a statistical trend towards increasing particle concentration

with increasing hemoglobin levels (r = 0.08; p = 0.05, Figure 6 B). There was no significant correlation between HDL particle concentration and hemoglobin (Figure 6 C).

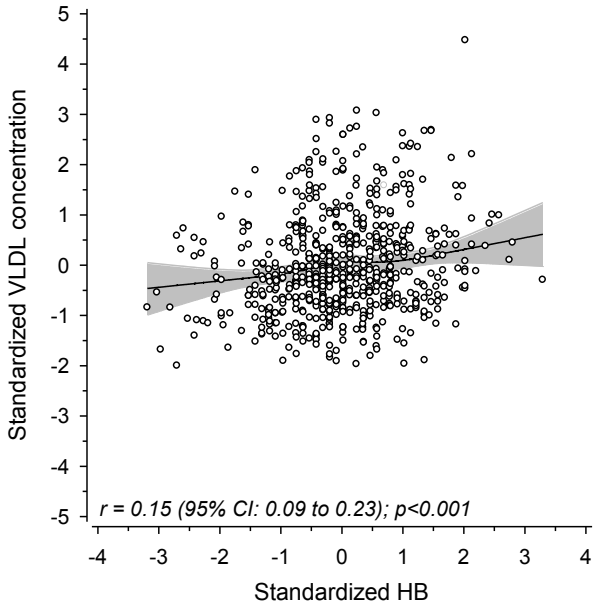

Figure 6 A. Correlation between hemoglobin and NMR measured VLDL particle concentration.

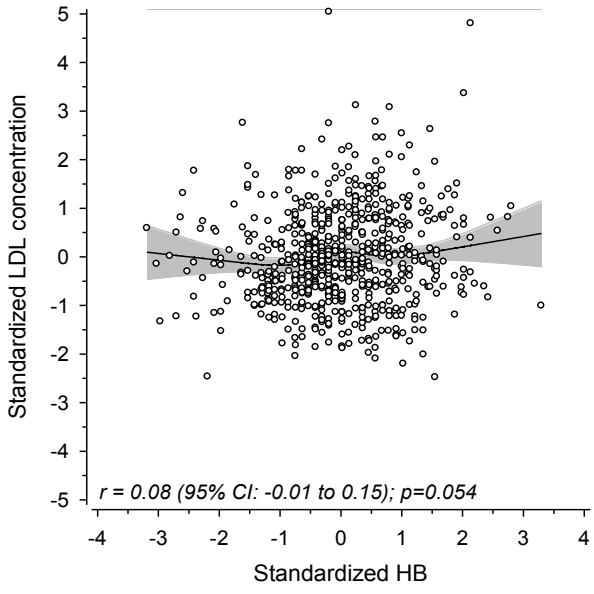

Figure 6 B. Correlation between hemoglobin and NMR-measured LDL particle concentration.

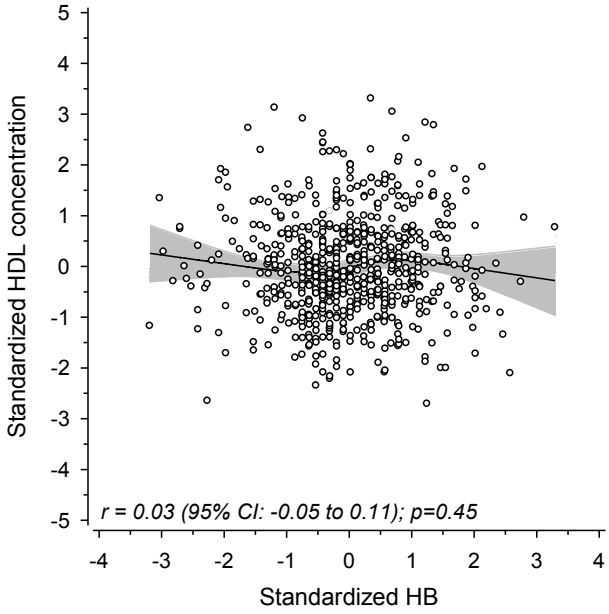

Figure 6 C. Correlation between hemoglobin and NMR-measured HDL particle concentration.

6 DISCUSSION

6.1 PRINCIPAL FINDINGS

Firstly, this study showed higher hemoglobin, erythropoietin, haptoglobin and ferritin levels in subjects with MetS and extended these findings to include individual MetS components.

Secondly, this study found an association between increased or reduced ferritin levels and MetS development or resolution, respectively, during a 6,5-year follow-up period. Also, the study showed significant changes in ferritin level in relation to resolving or developing the glucose, triglyceride and waist components of MetS.

Thirdly, this thesis demonstrated an association between higher hemoglobin levels with larger VLDL, smaller LDL and smaller HDL particle sizes as well as increasing concentrations of VLDL and LDL particles.

6.2 FINDINGS IN RELATION TO EARLIER RESEARCH

6.2.1 Erythropoietin, hemoglobin and haptoglobin level in subjects MetS

Previous studies have shown reduced adipose tissue oxygenation in obese subjects compared to normal-weight subjects and increased expression of EPO gene transcription stimulating factor (HIF) in the adipose tissue of obese subjects (Pasarica et al. 2009, Regazzetti et al. 2009, Wood et al. 2009).

Erythropoietin levels in subjects with MetS have not been investigated earlier. This study (I) reveals that erythropoietin levels were significantly higher in subjects with MetS as well as in subjects with the MetS abdominal obesity component, which may suggest underlying adipose tissue hypoxia and dysfunction in MetS. This is supported by a later study that found higher erythropoietin concentrations associated with larger fat body mass (Reinhardt et al. 2016).

In study I hemoglobin levels were significantly higher in subjects with MetS or with any of the components of MetS. Previously, higher hemoglobin concentrations have been independently associated with insulin resistance and incident type 2 diabetes (Barbieri et al. 2001, Choi et al. 2003, Tulloch-Reid et al. 2004). Also, a previous study of Thai subjects has shown increased hemoglobin concentrations with increasing numbers of MetS components but only in women (Lohsoonthorn et al. 2007). The mechanism that could explain the association between hemoglobin levels and MetS is unclear. Previously, hyperinsulinemia in insulin resistance has been suggested to promote erythrocytosis because endogenous insulin has a synergistic effect together with erythropoietin on stimulating the production of erythrocytes (Bersch et al. 1982). Later, visceral adiposity has been postulated as one of the underlying factors (Kawamoto et al., 2011, Kuthu et al. 2009). Visceral adiposity has been associated with hemoglobin level and the association between hemoglobin level and insulin resistance has been dependent on the level of visceral adiposity (Tabara et al. 2013).

Haptoglobin levels in subjects with MetS have not been previously investigated. This study (I) shows higher serum haptoglobin levels in subjects with MetS and subjects with an elevated glucose or blood pressure component even after adjusting for hs-CRP to minimize the impact of inflammation. Haptoglobin expression in adipocytes has been previously shown with increased levels in obesity and circulating haptoglobin levels have been positively correlated with body mass index and the level of adiposity (Chiellini et al. 2004, Friedrics et al. 1995). Haptoglobin concentration can increase during inflammation in response to cytokines (Levy et al. 2010). Thus, inflammation and cytokines are possible triggers of haptoglobin production in white adipose tissue (Friedrics et al. 1995). Though in this study (I) adjusting for hs-CRP did not change the association between haptoglobin and MetS, it is possible that other connecting mechanisms exists.

6.2.2 Transferrin receptor and ferritin levels in subjects with MetS

In study I, serum transferrin receptor (TFR) levels did not differ between subjects with or without MetS, but TFR levels were higher in subjects with the abdominal obesity component of MetS. No previous studies have investigated an association between TFR levels and MetS. Later, in a study of 725 Croatian adults, serum TFR levels were not associated with MetS or its components, but were positively associated with insulin resistance (Suarez-Ortegon et al. 2016). In study I insulin resistance was not included in the adjusted model and it could have had an impact on a significant association between TFR levels and abdominal obesity. However, a positive association between abdominal obesity and TFR level is in line with later studies that have found higher TFR levels in obese subjects (Zhao et al. 2015). Also, in a later study, subjects with obesity and high waist circumference, elevated TFR levels were associated with an increased risk of developing type 2 diabetes (Fernandez-Cao et al. 2017).

Study I showed that subjects with MetS had significantly higher serum ferritin levels. That is supported by results of previous cross-sectional studies that consistently have reported higher ferritin levels in subjects with MetS (Jehn et al. 2004, Kim et al. 2011, Lee et al. 2011, Ryoo et al. 2011, Sun et al. 2008, Shi et al. 2008). This is also supported by later cross-sectional studies (Abril-Ulloa et al. 2014, Chang et al. 2012, Chen et al. 2017, Kilani et al. 2014, Ledesma et al. 2015, Li et al., 2013, Suarez-Ortegon et al. 2018). In addition, In Study I, ferritin levels were significantly higher in subjects with abdominal obesity or high triglycerides or an elevated glucose component and also significantly but less in subjects with the low HDL cholesterol MetS component. No association was found between the blood pressure component and ferritin levels. This is supported by a later meta-analysis, which reported stronger associations between ferritin level and high triglycerides or high fasting glucose than with other components of MetS (Suarez-Ortegon et al. 2018).

6.2.3 Ferritin level and development or resolution of MetS and its components

Study II showed that an increase in serum ferritin levels during the 6.5-year follow-up period was significantly higher in men and in women with incident MetS compared with men and women without development of MetS. Three previous longitudinal studies have evaluated the association between baseline serum ferritin level and development of MetS during a follow-up time of 3 to 6 years (Park et al. 2012, Vari et al. 2007, Yoon et al. 2012).

According to the number of incident cases of MetS, compared to Study II, one larger study has been previously done by Park et al. in year 2012. The study by Park et al. showed an association between ferritin level and incident MetS in men, thus including only male subjects (Park et al. 2012). Smaller previous studies supported the results of Study II and found an association between higher ferritin levels and incident MetS in women and men (Vari et al. 2007, Yoon et al. 2012). Later, a study by Kilani et al. did not find a significant association between ferritin and incident MetS either in women or men in an adjusted model that, unlike in other studies, was also included BMI (Kilani et al. 2015). In study II, results were adjusted for age, baseline smoking, baseline physical activity, baseline use of alcohol, baseline serum ferritin level, baseline hs-CRP and also baseline BMI and a significant association was still found between increasing ferritin level and incident MetS in the 6.5-year follow-up time. In Study II, the change in ferritin level during the follow-up time was used instead of baseline ferritin, unlike in previous studies, which also made it possible to evaluate the association for the resolution of MetS during the follow-up. It was found that ferritin levels increased significantly less in women in whom the criteria for MetS resolved during the follow-up period compared with women in whom the MetS criteria remained.

The decrease or increase in serum ferritin was significantly higher in men whose triglyceride criterion for MetS resolved or developed over the follow-up period (Study II). Also, a positive correlation between a change in serum ferritin level and change in plasma triglyceride was significant in men ($p < 0.001$). One of the previous longitudinal studies has estimated the development of different MetS components in association with baseline ferritin levels (Vari et al. 2007). Supporting the result of Study II, it was found that hypertriglyceridemia in the follow-up was most strongly associated with baseline ferritin levels both in men and women (Vari et al. 2007). However, it did not find associations between other MetS components and baseline ferritin. Study II also showed significantly less increased serum ferritin levels in women whose glucose criterion for MetS resolved during the follow-up period and also a significant positive correlation

between a change in serum ferritin level and glucose level in men. In addition, serum ferritin levels increased significantly more in women whose waist criterion for MetS developed during the follow-up period. Also, there was a significant positive correlation between a change in serum ferritin level and a change in waist circumference both in men and women even in an adjusted model that included BMI. Waist circumference was the only one of the Mets components that was positively associated with a change in ferritin level both in men and women, which may indicate the importance of waist circumference in the development of Mets.

6.2.4 Lipoprotein particle concentration and size in relation to hemoglobin level

In Study III, larger VLDL particle size, smaller LDL particle size, and smaller HDL particle size were associated with higher hemoglobin concentrations. Also, increasing VLDL particle as well as increasing LDL particle concentration was associated with higher hemoglobin level. No previous study has evaluated the association between lipoprotein particle sizes or concentrations and hemoglobin level. However, previous studies have evaluated associations between NMR-measured lipoprotein particles sizes and impaired glucose tolerance, type 2 diabetes and metabolic syndrome (Abbasi et al. 2013, Austin et al. 1995, Fagot-Campagna et al. 1999, Fizelova et al. 2015, Frazier-Wood et al. 2011, Mora et al. 2010, Wang et al. 2012).

In cross-sectional studies, individuals with impaired fasting glucose or glucose tolerance have had increased concentrations of VLDL subclass particles, especially larger VLDL particles, and decreased concentrations of larger HDL particles (Wang et al. 2012). In prospective studies, increased concentrations of large HDL particles have shown to be preventive for hyperglycemia and incident type 2 diabetes (Abbasi et al. 2013, Fagot-Campgna et al. 1999, Fizelova et al, 2015). On the contrary, increased levels of small HDL, small LDL and large VLDL particles have been shown to be associated with increased risk of developing hyperglycemia and type 2 diabetes (Austin et al. 1995, Fagot-Campagna et al. 1999, Fizelova et al. 2015, Mora et al. 2010). Metabolic syndrome and its individual components have been characterized by a reduction in LDL and HDL particle sizes (Frazier-Wood et al. 2011, Kathiresan et al. 2006). Larger VLDL particles have been associated especially high glucose or diabetes, triglycerides and waist circumference components of MetS (Frazier-Wood et al. 2011).

The results in study III were adjusted for HOMA-IR to evaluate the influence of insulin resistance associated with type 2 diabetes and metabolic syndrome. Adjusting did not change the results significance except the association of VLDL particle size and hemoglobin in women. This suggests that insulin resistance mostly affects the VLDL particles, but there exist also other mechanisms affecting the relation of lipoprotein particle size and hemoglobin level. Also, because of the previous finding that ferritin is strongly associated with MetS, the association between serum ferritin level and lipoproteins VLDL, LDL and HDL particle sizes were investigated, but no significant associations were found.

Associations of larger VLDL, smaller LDL and smaller HDL particle size with higher hemoglobin concentrations remained unchanged after adjusting for concentrations of VLDL, LDL, or HDL particles. In addition, increasing VLDL particle concentration as well as increasing LDL particle concentration was associated with higher hemoglobin concentration, although the associations were weaker than with particle sizes. These findings suggest that lipoprotein particle size and concentration have both their own independent association to hemoglobin level.

6.3 STRENGTHS AND LIMITATIONS

6.3.1 Study population, methods, design

The strength of this thesis is the relatively large study population, which consisted of five entire age groups from one town (born in 1942, 1947, 1952, 1957 and 1962, n=1294) with no exclusion criteria. Thus, the study population represents a mostly genetically homogenous middle-aged (mean age 52 years) Caucasian population. The number of participants at the baseline visit was representative (n = 923, 71%) as well as in the follow-up visit (n=766, 59%). Among the population of the follow-up visit, 681 were the same subjects as in the baseline visit (74%). Biochemical measurements, prevalence of the metabolic syndrome, and use

of medication for hypertension, diabetes, and dyslipidemia did not differ between subjects who participated in both health care visits compared to subjects who did not participate in the follow-up visit.

Subjects with MetS were older compared to subjects without Mets (Study I and II). However, all results were age adjusted. Smoking habits between subjects with and without MetS did not differ significantly. In addition, all results were adjusted for smoking to exclude its influence particularly on hemoglobin and erythropoietin levels. Current use of alcohol was higher in female subjects without MetS. Alcohol use can influence ferritin levels in particular, and all results in Study II were adjusted for baseline alcohol use. In study II, the relatively small number of MetS cases that resolved during the 6.5-year follow-up period (n=44, 6%) is a limitation, and could have affected the non-significant results observed in men. All subjects using medications for dyslipidemia and diabetes are included in analyses. In studies I and II, use of medication is part of the definition of MetS. Subjects using medication for dyslipidemia were classified as having both the HDL and triglyceride components of MetS. In study III, the analyses were not adjusted for medication, which could have affected the results.

The cross-sectional study design in Studies I and III does not allow us to make conclusions about causality either of the relationships of hypoxia markers with the MetS nor the associations between lipoprotein particle sizes and hemoglobin level. That is because hematological parameters were measured only from follow-up visits in years 2003-2004. However, in Study II it was possible to utilize the longitudinal study design with the iron metabolism marker ferritin.

All results (Studies I, II, II) were hs-CRP adjusted to estimate the impact of inflammation. That is particularly important in analyses of acute phase proteins haptoglobin and ferritin levels in Studies I and II. However, we were not able to estimate other markers of inflammation or levels of proinflammatory cytokines.

Information about women's menopause status was not available and this could affect women´s hemoglobin, erythropoietin, haptoglobin and ferritin levels (Study I). Mean ferritin levels are known to be higher in postmenopausal women compared to premenopausal women (Milnam et al., 1996). To exclude this, a separate analysis was done in women with age adjustment. The age adjustment did not alter the results in women.

Unfortunately, it was not possible to evaluate the possible impact of obstructive sleep apnea on a subject´s hemoglobin or erythropoietin levels. Previous studies have reported higher serum erythropoietin concentrations in association with increased nocturnal hypoxia time and also decreasing serum EPO concentrations with positive pressure airway treatment in patients with obstructive sleep apnea (Fleming et al. 2018, Fleming et al. 2016).

It was not possible to exclude persons with gene mutations of the most common iron storage disease, hemochromatosis. All baseline ferritin levels were less than 730 μg/l (range, 2–722 μg/l), but that does not exclude especially a combination of heterozygous mutations, which are quite common in the Caucasian population. A combination of heterozygous mutations of hemochromatosis does not necessarily cause iron overload or high ferritin levels without other predisposing factors such as metabolic syndrome (Beckman et al. 1997, Feder et al. 1996). In order to exclude hemochromatosis, it is primarily recommended to determine transferrin-iron saturation, which was not included in this study (European Association For The Study Of The Liver 2010).

Additionally, it was not possible to exclude the impact of non-alcoholic fatty liver disease (NAFLD) in this study. A mildly elevated ferritin level is a common feature of NAFLD as well as elevated alanine aminotransferase (ALAT) and gamma-glutamyl transferase (GT) levels (Chalasani et al. 2017, Kowdley et al. 2012). In this study male and female subjects with MetS had higher ALAT ja GT levels compared to subjects without and it is possible that NAFLD is one influencing factor in that difference.

It was not possible to exclude other liver diseases like liver cirrhosis and cancer that can alter ferritin levels. However, all baseline ALAT levels were less than 120 U/L (range, 4-120 U/L) and the 6.5-year follow-up period make these liver diseases unlikely. Additionally, information about the nutritional content of subjects' diets or their consumption of dietary supplements like iron or antioxidants were not available.

6.4 FUTUTE IMPLICATIONS

Simple and affordable markers for clinical medicine are needed for the detection of high metabolic risk patients. This study brings new evidence that markers of adipose tissue dysfunction and iron metabolism are associated with metabolic risk factors: metabolic syndrome, its components and an atherogenic lipoprotein profile. This study provides new markers to evaluate the risk and development of metabolic syndrome. Further, at the population level, the findings of this study can suggest new parameters for evaluating the outcome of the prevention and treatment of metabolic syndrome.

Further longitudinal studies could provide more information about causal relationships of adipose tissue dysfunction markers like hemoglobin and erythropoietin as well as comprehensive iron metabolism markers and the progression of cardiovascular diseases and cardiovascular mortality. Also, further research is needed to estimate these markers in order to evaluate the efficacy of prevention and treatment interventions in metabolic syndrome, type 2 diabetes and cardiovascular disease.

7 SUMMARY AND CONCLUSIONS

The prevalence of metabolic syndrome (MetS) is increasing with the aging of the population and the prevalence of obesity. More information is needed on the factors affecting the progression of MetS and its transition to cardiovascular disease as well as on the markers that could help recognize the subjects at high risk in clinical work.

This study brings new information about the associations of markers of adipose tissue dysfunction and iron metabolism with MetS and information about the association of an atherogenic lipoprotein profile with hemoglobin level.

The major findings are that subjects with metabolic syndrome have elevated hemoglobin, erythropoietin, haptoglobin and ferritin levels. Higher hemoglobin levels are related to all the components of MetS. Higher haptoglobin levels are related to hyperglycemia and blood pressure components whereas erythropoietin levels are related only with the abdominal obesity component. Higher hemoglobin levels are also associated with an atherogenic lipoprotein particle profile: with larger VLDL, smaller LDL, and smaller HDL particle sizes and increasing amounts of larger VLDL and smaller LDL particles. An increase in serum ferritin levels in a 6.5-year follow-up period is associated with the development of MetS in both men and women.

In conclusion, the findings of this study suggest that erythropoietin concentrations can act as a marker for adipose tissue dysfunction associated with MetS. Hemoglobin is a readily available laboratory parameter that could complement the risk assessment of a patient with metabolic risk factors possibly suggesting a higher CVD risk profile. Ferritin levels can act as a marker for the detection of MetS as well as in the follow-up of patients with MetS or its components. The limitations concerning the use of ferritin are other conditions influencing its levels and should be taken into account.

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ORIGINAL PUBLICATIONS (I - III)

I

Erythropoietin, ferritin, haptoglobin, hemoglobin and transferrin receptor in metabolic syndrome: a case control study.

Hämäläinen P, Saltevo J, Kautiainen H, Mäntyselkä P and Vanhala M.

Cardiovasc Diabetol. Sep 27; 11: 116, 2012 doi: 10.1186/1475-2840-11-116.

CARDIO VASCULAR DIABETOLOGY

ORIGINAL INVESTIGATION Open Access

Erythropoietin, ferritin, haptoglobin, hemoglobin and transferrin receptor in metabolic syndrome: a case control study

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Abstract

Background: Increased ferritin concentrations are associated with metabolic syndrome (MetS). The association between ferritin as well as hemoglobin level and individual MetS components is unclear. Erythropoietin levels in subjects with MetS have not been determined previously. The aim of this study was to compare serum erythropoietin, ferritin, haptoglobin, hemoglobin, and transferrin receptor (sTFR) levels between subjects with and without MetS and subjects with individual MetS components.

Methods: A population based cross-sectional study of 766 Caucasian, middle-aged subjects (341 men and 425 women) from five age groups born in Pieksämäki, Finland who were invited to a health check-up in 2004 with no exclusion criteria. Laboratory analyzes of blood samples collected in 2004 were done during year 2010. MetS was defined by National Cholesterol Education Program criteria.

Results: 159 (53%) men and 170 (40%) women of study population met MetS criteria. Hemoglobin and ferritin levels as well as erythropoietin and haptoglobin levels were higher in subjects with MetS ($p < 0.001$, $p = 0.018$). sTFR level did not differ significantly between subjects with or without MetS. Hemoglobin level was significantly higher in subjects with any of the MetS components ($p < 0.001$, $p = 0.002$). Ferritin level was significantly higher in subjects with abdominal obesity or high TG or elevated glucose or low high density cholesterol component ($p < 0.001$, $p = 0.002$, $p = 0.02$). Erythropoietin level was significantly higher in subjects with abdominal obesity component ($p = 0.015$) but did not differ significantly between subjects with or without other MetS components. Haptoglobin level was significantly higher in subjects with blood pressure or elevated glucose component o MetS ($p = 0.028$, $p = 0.025$).

Conclusion: Subjects with MetS have elevated hemoglobin, ferritin, erythropoietin and haptoglobin concentrations. Higher hemoglobin levels are related to all components of MetS. Higher ferritin levels associate with TG, abdominal obesity, elevated glucose or low high density cholesterol. Haptoglobin levels associate with blood pressure or elevated glucose. However, erythropoietin levels are related only with abdominal obesity. Higher serum erythropoietin concentrations may suggest underlying adipose tissue hypoxemia in MetS.

Keywords: Erythropoietin, Ferritin, Hemoglobin, Metabolic syndrome

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Metabolic syndrome (MetS) is a pathophysiological disorder with clustering of risk factors -abdominal obesity, increased blood pressure, glucose intolerance and dyslipidemia - for cardiovascular disease and type 2 diabetes [1,2]. Previous studies have reported alterations in hematological parameters and iron metabolism: a trend towards higher hemoglobin concentrations and serum ferritin levels in subjects with MetS [3-6]. However, the association between hemoglobin as well as ferritin level and individual MetS components is still unclear.

Erythropoietin (EPO) is a glycoprotein hormone whose production in kidneys is stimulated by hypoxia and it is a known stimulator of erythrocyte production and hemoglobin synthesis [7]. Recently, increasing amount of evidence has suggested that reduced adipose tissue oxygenation and cellular hypoxia may be an underlying cause of adipose tissue dysfunction contributing to metabolic changes like insulin resistance associated with obesity and MetS [8-10]. Erythropoietin levels in subjects with MetS have not been determined previously.

The aim of this study was to compare serum haptoglobin, hemoglobin, ferritin, erythropoietin and transferrin receptor levels between subjects with and without MetS and extend these findings to include individual MetS components.

Research design and methods

Study sample

The study population primarily consisted of 1294 middle-aged subjects from Pieksämäki, Finland, who were born in 1942, 1947, 1952, 1957 or 1962 and invited to a health check-up in the years 1997–1998 initially and to a follow-up check-up in 2004. A total of 923 out of 1294 subjects participated in the initial examination in 1997–1998 and 766 of these participated in a second health check-up in 2003–2004 when hematological laboratory tests were taken. These hematological laboratory tests were analyzed in Kuopio University laboratory during year 2010.

The final analysis included data from these 766 subjects. The study protocol was approved by the Ethics Committee of Kuopio University Hospital and the University of Eastern Finland. All participants gave informed written consent.

All subjects filled in a questionnaire about their smoking habits, alcohol consumption, and physical activity. They were also interviewed by a trained nurse. Subjects who smoked daily were considered to be current smokers. Alcohol consumption was divided into three categories: low, meaning no alcohol use; moderate (less than two portions of alcohol per day); and high (more than two portions per day). Physical activity was considered to be high in subjects who exercised daily at least 30 minutes in their leisure time, moderate in subjects who exercised at least three times per week, and low if exercising frequency was less than three times per week [11].

Two trained nurses performed the study processing and physical examination. Blood pressure was measured with a mercury sphygmomanometer in a sitting position after 15 minutes of rest. The measurement was repeated after five minutes. The mean of the two measurements was used in the statistical analyses. Waist circumference was measured from the midpoint between the lateral iliac crest and the lowest rib to an accuracy of 0.5 cm.

MetS was defined by the updated National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (ATP III) criteria [12]. Subjects with three or more of the following components were classified as having MetS: (1) increased waist circumference (≥102 cm (≥40 in) for men and ≥88 cm (≥35 in) for women); (2) elevated fasting total triglycerides (≥1.7 mmol/l (≥150 mg/dl) or treatment for dyslipidemia); (3) low fasting serum high density lipoprotein (HDL) cholesterol (<1.03 mmol/l (<40 mg/dl) in men or <1.29 mmol/l (<50 mg/dl) in women or treatment for dyslipidemia); (4) systolic blood pressure ≥130 mmHg or diastolic blood presure ≥85 mmHg or the use of antihypertensive medication; and (5) fasting plasma glucose ≥5.6 mmol/l (≥100 mg/dl) or the use of antihyperglycemic medication.

Laboratory methods

Fresh serum samples were drawn after an overnight fast. Plasma was separated by centrifugation for the determination of glucose and lipids and the samples were frozen immediately and stored at - 70°C.

Samples were analyzed in Kuopio regional laboratory during year 2010.

Plasma glucose concentration was measured by an automated colorimetric method (Peridochrom Glucose GOD-PAP, Boehringer, Germany). Serum triglycerides were measured from fresh serum samples by enzymatic colorimetric methods (CHOD-PAP, GPO-PAP, Boehringer Mannheim GmbH, Germany). Serum HDL cholesterol was measured by the same method after precipitation of low-density lipoprotein cholesterol and very low-density lipoprotein cholesterol with phosphotungestic acid and magnesium. High-sensitivity C-reactive protein (hs-CRP) was measured with an Immulite analyzer and a DPC high-sensitivity CRP assay (DPL, Los Angeles, CA, USA).

White blood cell and platelet count, hemoglobin and hematocrit were measured using an automatic electronic cell calculator. Serum EPO was analyzed using an immunoluminometric assay method. Serum soluble transferrin receptor concentration was measured by a particle enhanced immunoturbidimetric assay (Cobas c systems, Roche Diagnostics GmbH, Mannheim, Germany). Serum

ferritin concentration was analyzed using an electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany).

The analytical method for plasma haptoglobin concentration measurements was an immunoturbidimetric assay (Cobas c systems, Roche Diagnostics GmbH, Mannheim, Germany, ACN 228). Serum creatinine was measured using an enzymatic method.

Statistical analyses

The results are expressed as means and standard deviations (SDs) for continuous variables and as proportions for categorical variables. The normality of variables was evaluated by the Shapiro-Wilk test. Statistical comparisons between the groups were performed using the chi-square test, *t*-test, or bootstrap-type *t*-test as appropriate. Bootstrap type analysis of covariance was also used to compare the groups as measurements. In these analyses age values, sex, smoking, physical activity and hs-CRP values were used as covariates. For all analyses, $P < 0.05$ was considered significant.

Results

The study population included 425 women and 341 men with a mean age of 52.1 ± 6.4 and 52.1 ± 6.2 years, respectively. MetS was present in 52% of women and in 48% of men. Table 1 shows the clinical and lifetime factors for subjects with and without MetS. Both women and men with MetS were significantly older than subjects without. Life-style factors did not differ significantly between subjects with or without MetS. In those with MetS, 14% of female and 26% of male subjects were classified as current smokers. In those without MetS, the proportions of female and male smokers to non-smokers were 19% and 25% respectively.

Blood pressure was the most common component of MetS. It was present in 78% of the men and 66% of the women. High fasting plasma glucose was also present in a large part of the subject (75% of the men and 53% of the men, Table 1).

Figures 1, 2, 3, 4 and 5 show standardized means for erythropoietin, ferritin, haptoglobin, serum transferrin receptor (sTFR) and hemoglobin in subjects with and without MetS and in subjects with or without an individual MetS component. All results are standardized for age, sex, smoking, physical activity and hs-CRP. Also adjustment for creatinine as a marker of renal function and for alcohol comsumption was done afterwards (data not shown). This did not change the results. Mean hemoglobin and mean ferritin were significantly higher in subjects with MetS ($p < 0.001$). Mean erythropoietin was significantly higher in men with MetS ($p = 0.006$) and remained significantly higher in subjects with MetS after adjusting for sex ($p = 0.018$). Mean haptoglobin was significantly higher in subjects with MetS ($p = 0.018$). Mean sTFR did not differ significantly between subjects with or without MetS.

Mean hemoglobin was significantly higher in subjects with any of the MetS components (abdominal obesity, blood pressure (BP), low HDL, high triglycerides (TG) or elevated glucose). Mean ferritin was significantly higher in subjects with abdominal obesity or low HDL or high TG or elevated glucose component. Mean erythropoietin was significantly higher in subjects with abdominal obesity component but did not differ significantly between subjects with or without other components of MetS. Mean haptoglobin was significantly higher in subjects with blood pressure or elevated glucose component. Mean sTFR was significantly higher in subjects with abdominal obesity component but did not differ significantly between subjects with or without other components of MetS.

Discussion and conclusion

To our knowledge, this is the first study to show higher hemoglobin, serum ferritin, haptoglobin and also erythropoietin levels in subjects with MetS and extending these findings to include individual MetS components.

Recently, increasing evidence has suggested that reduced adipose tissue oxygenation and cellular hypoxia may be an underlying cause of adipose tissue dysfunction contributing to metabolic changes associated with obesity and MetS [8-10]. It was demonstrated that hypoxia creates an insulin resistant state in human adipocytes by inhibiting phosphorylation of the insulin receptor, leading to a decrease in glucose transport [10]. Insulin resistance has been the most accepted and unifying hypothesis to describe the pathophysiology of the metabolic syndrome [1].

Also, previous studies have shown reduced adipose tissue oxygenation in obese compared to normal-weight subjects and EPO gene transcription stimulating factor (HIF-1) over-expression in the adipose tissue of obese subjects [8] [13]. Hypoxia is a known stimulator of erythropoietin production as well as EPO is a stimulator of hemoglobin synthesis [7]. EPO levels were significantly higher in subjects with MetS as well as in subjects with MetS abdominal obesity component in this study which may suggest underlying adipose tissue hypoxia in MetS.

Hemoglobin levels were significantly higher in subjects with MetS or with any of the components of MetS. This is supported by a previous study of working-age thai subjects that showed increased hemoglobin concentrations with increasing numbers of MetS components but only in women [14].

Further research is needed to investigate possible association between higher hemoglobin and EPO levels in subjects with MetS.

Table 1 Clinical and life-style characteristics of the study population in subjects with and without the MetS

Characteristics	Male		p	Female		p
	MetS present N = 159	MetS not present N = 182		MetS present N = 170	MetS not present N = 255	
Age, years **	53.7 (5.8)	50.7 (6.2)	<0.001	54.4 (5.7)	50.5 (6.4)	<0.001
Body mass index **	29.8 (3.9)	25.2 (2.5)	<0.001	30.8 (5.3)	25.1 (3.5)	<0.001
Waist, cm **	103.8 (10.7)	89.6 (7.2)	<0.001	96.2 (12.4)	81.0 (8.5)	<0.001
FP-gluc mmol/L **	6.5 (1.4)	5.8 (0.8)	<0.001	6.3 (1.5)	5.5 (0.4)	<0.001
BP systolic, mmHg **	143 (19)	136 (17)	<0.001	144 (17)	131 (16)	<0.001
BP diastolic, mmHg **	87 (9)	82 (10)	<0.001	86 (9)	79 (8)	<0.001
HDL-C, mmol/L **	1.3 (0.4)	1.6 (0.4)	<0.001	1.6 (0.4)	1.8 (0.3)	<0.001
Trigly, mmol/L **	1.9 (1.5)	1.1 (0.5)	<0.001	1.6 (0.8)	1.0 (0.3)	<0.001
Creatinine μmol/L **	88.5 (11.9)	85.9 (8.7)	0.02	75.3 (14.8)	73.8 (8.9)	0.17
Hemoglobin (g/L) **	154 (9)	150 (9)	<0.001*	141 (10)	136 (9)	<0.001*
Ferritin (μg/L) **	216 (165)	151 (112)	<0.001*	94 (75)	61 (48)	<0.001*
TFR (mg/L) **	2.9 (2.8)	2.6 (0.6)	0.12*	2.8 (0.9)	2.7 (1.0)	0.32*
Haptoglobin (g/L) **	1.3 (0.6)	1.1 (0.5)	0.012*	1.4 (0.6)	1.2 (0.4)	<0.001*
Hs-CRP (mg/L) **	2.4 (3.5)	1.5 (2.9)	0.058*	3.1 (3.4)	1.5 (2.3)	<0.001*
Components of MetS:						
Waist n (%)	102 (64)	8 (4)	<0.001	134 (79)	40 (16)	<0.001
FP-glucose n (%)	144 (91)	113 (62)	<0.001	139 (83)	85 (33)	<0.001
Blood pressure n (%)	146 (92)	119 (65)	<0.001	154 (90)	126 (49)	<0.001
HDL-cholesterol n (%)	90 (57)	2 (1)	<0.001	101 (59)	10 (4)	<0.001
Triglycerides n (%)	116 (73)	16 (9)	<0.001	118 (69)	6 (2)	<0.001
Life-style factors, n (%):						
Current smoker	42 (26)	46 (25)	0.81	25 (14)	49 (19)	0.23
Current use of alcohol						0.038
Low (nothing)	20 (12)	26 (14)	0.055	46 (28)	46 (18)	
Moderate	68 (43)	97 (54)	0.055	97 (58)	177 (70)	
High	71 (45)	58 (32)	0.055	24 (14)	31 (12)	
Physical activity n (%)						0.83
Low	33 (21)	56 (31)	0.11	55 (33)	80 (32)	
Moderate	97 (61)	96 (53)	0.11	92 (55)	146 (57)	
High	29 (18)	30 (16)	0.11	21 (12)	28 (11)	

*Values are adjusted for age.
** Mean (SD).
Abbreviations: Fp-gluc, fasting plasma glucose; BP systolic, systolic blood pressure; BP diastolic, diastolic blood pressure; HDL-C, high density cholesterol; Trigly, triglycerides; TFR, transferrin receptor; Hs-CRP, high sensitivity c-reactive protein.
Components of MetS.
1. Waist >102 cm (male) or >88 cm (female).
2. FP-glucose ≥ 5.6 mmol/L.
3. Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication.
4. HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia.
5. Triglycerides >1.7 mmol/l or medication for dyslipidemia.
Current use of alcohol was consireded low with no use of alcohol, moderate with use of <2 portions/day and high with use of >2 portions/day. Physical activity was considered to be high in subjects who exercised daily at least 30 minutes in their leisure time, moderate in subjects who exercised at least three times per week, and low if exercising frequency was less than three times per week.

Previously, it was shown that nocturnal intermittent hypoxia, a marker for obstructive sleep apnea (OSA), is positively associated with MetS and its components [15]. One previous study reported lower serum EPO concentrations after continuous positive pressure airway treatment in patients with OSA [16]. Also, higher serum EPO concentrations have been reported in patients with central sleep

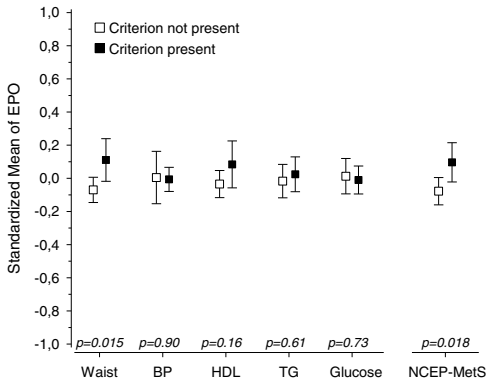

Figure 1 Eryhtropoietin mean values and individual MetS components. All values are standardized for age, sex, hs-CRP, smoking and physical activity. Abbreviations: BP, blood pressure; EPO, erythropoietin; HDL, high density lipoprotein cholesterol; TFR, transferrin receptor; TG, triglycerides. NCEP (National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults) criteria of MetS: Waist >102 cm(male) or >88 cm(female); FP-glucose ≥ 5.6 mmol/L; Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication; HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia; triglycerides >1.7 mmol/l or medication for dyslipidemia.

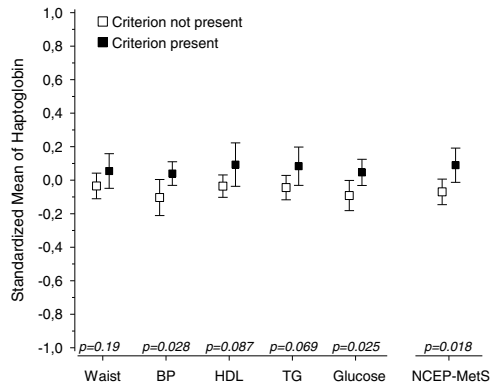

Figure 3 Haptoglobin mean values and individual MetS components. All values are standardized for age, sex, hs-CRP, smoking and physical activity. Abbreviations: BP, blood pressure; EPO, erythropoietin; HDL, high density lipoprotein cholesterol; TFR, transferrin receptor; TG, triglycerides. NCEP (National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults) criteria of MetS: Waist >102 cm(male) or >88 cm(female); FP-glucose ≥ 5.6 mmol/L; Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication; HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia; triglycerides >1.7 mmol/l or medication for dyslipidemia.

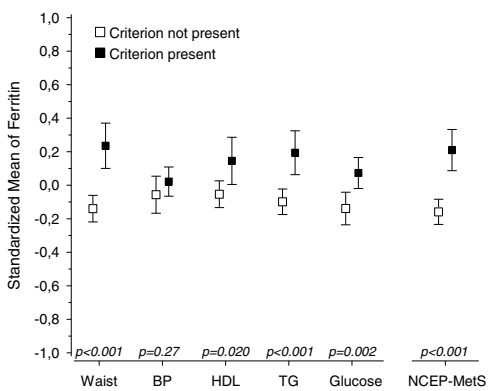

Figure 2 Ferritin mean values and individual MetS components. All values are standardized for age, sex, hs-CRP, smoking and physical activity. Abbreviations: BP, blood pressure; EPO, erythropoietin; HDL, high density lipoprotein cholesterol; TFR, transferrin receptor; TG, triglycerides. NCEP (National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults) criteria of MetS: Waist >102 cm(male) or >88 cm(female); FP-glucose ≥ 5.6 mmol/L; Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication; HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia; triglycerides >1.7 mmol/l or medication for dyslipidemia.

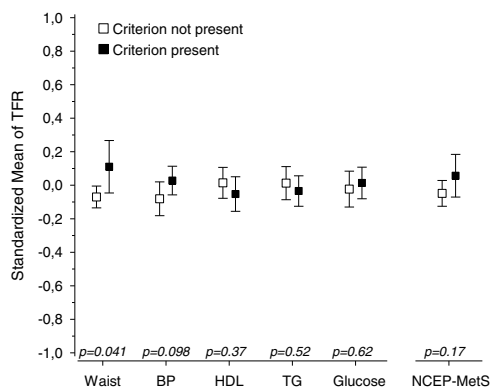

Figure 4 Transferrin receptor mean values and individual MetS components. All values are standardized for age, sex, hs-CRP, smoking and physical activity. Abbreviations: BP, blood pressure; EPO, erythropoietin; HDL, high density lipoprotein cholesterol; TFR, transferrin receptor; TG, triglycerides. NCEP (National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults) criteria of MetS: Waist >102 cm(male) or >88 cm(female); FP-glucose ≥ 5.6 mmol/L; Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication; HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia; triglycerides >1.7 mmol/l or medication for dyslipidemia.

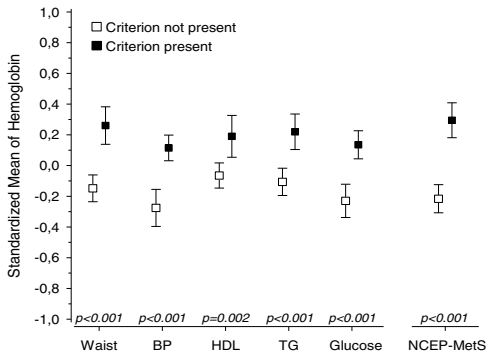

Figure 5 Hemoglobin mean values and individual MetS components. All values are standardized for age, sex, hs-CRP, smoking and physical activity. Abbreviations: BP, blood pressure; EPO, erythropoietin; HDL, high density lipoprotein cholesterol; TFR, transferrin receptor; TG, triglycerides. NCEP (National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults) criteria of MetS: Waist >102 cm(male) or >88 cm(female); FP-glucose ≥ 5.6 mmol/L; Systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg or antihypertensive medication; HDL–cholesterol < 1.03 mmol/l (men) or < 1.29 mmol/l (women) or medication for dyslipidemia; triglycerides >1.7 mmol/l or medication for dyslipidemia.

apnea and nocturnal hypoxia compared to healthy controls [17].

Previous studies have shown associations between serum ferritin or sTFR and increased risk of type 2 diabetes [18-21]. Recently, it was also shown that single nucleotide polymorphism (SNP) in genes that are related to body iron status are associated with risk of type 2 diabetes (T2D). SNP in gene that was related high sTFR levels and low ferritin levels was associated with lower risk of T2D, as well [20].

The finding that subjects with MetS had significantly higher serum ferritin levels supports previous results [3-6]. In addition, ferritin levels were significantly higher in subjects with abdominal obesity or high TG or elevated glucose or low high-density cholesterol MetS component but not in subjects with blood pressure component. Previous studies have shown that higher serum ferritin concentrations are associated with increased TG concentration in men and with elevated glucose in women [3,4].

Because ferritin is an acute phase reactant, all results were adjusted for hs-CRP to estimate the impact of inflammation. Ferritin levels remained significantly higher after hs-CRP standardization suggesting that mechanisms other than inflammation may be influencing ferritin concentration in the subjects with MetS. However, we were not able to estimate other markers of inflammation or level of proinflammatory cytokines like tumor necrosis factor alfa and interleukins in this study.

Higher hs-CRP levels have also previously shown to be associated with MetS and its separate components as well as median hs-CRP levels to be increased with increasing number of MetS components [21-24]. In addition, the degree of central obesity seemed to be the main determinant of an increased hs-CRP level [24]. In our study hs-CRP levels were significantly higher in women with MetS and almost significantly higher in men with MetS compared those without (Table 1).

Under hypoxia and also when erythropoiesis is stimulated, human iron-regulatory hormone, hepcidin, production is suppressed [25,26]. Theoretically, suppression in hepcidin production could result in higher ferritin levels seen in subjects with MetS. However, althought reduced adipose tissue oxygenation was found in obese subjects, hepcidin expression levels were increased, not suppressed, in the adipose tissue of obese patients [27]. Consequently, it is unlikely that purely adipose tissue hypoxia could cause hepcidin supression and elevated ferritin levels.

Haptoglobin is an acute phase reactant which plasma levels are increased during inflammation [28]. Although the liver is the major source of haptoglobin, research has demonstrated that it is also secreted into plasma by adipose tissue [29]. Serum haptoglogin level was previously shown to be positively associated with body fat [30]. Our study shows higher serum haptoglobin levels in subjects with MetS and subjects with elevated glucose or blood pressure component even after adjusting for hs-CRP.

Serum transferrin receptor levels did not differ between subject with or without MetS, but sTFR level was higher in subjects with abdominal obesity component of MetS. sTFR levels are increased in iron defiency with inadequate iron supply for erythropoiesis [31] but also, for example secondary to use of erythropoiesis stimulating agents such as erythropoietin [32]. Higher sTFR levels in subjects with abdominal obesity component of MetS suggest that despite of higher ferritin levels, these subjects are not iron overloaded. Previous studies have shown higher sTFR leves in obese subjects as well as no iron accumulation in liver biopsies of obese patients [27,33].

The strength of our study is the study population with five age groups and no exclusion criteria. Smoking habits between subjects with and without MetS did not differ significantly. In addition, all results were adjusted for smoking to exclude its influence particularly on hemoglobin and erythropoietin levels. All results were also hs-CRP adjusted to estimate the impact of inflammation. A limitation is that hematological parameters were measured only at the second health check-up and cross-sectional study design does not allow identification

of proper causal relationships. Information about women's menopause status was not available. However, the separate analysis was done in women for age adjusment (Table 1, all data not shown). The age adjusment did not affect the results in women. Unfortunately, we were unable to evaluate a possibly impact of obstructive or central sleep apnea on subjects hemoglobin or erythropoietin levels. Also, information about nutritional content of subjects' diets or consumption of dietary supplements like iron or antioxidants was not available.

In conclusion, Subjects with MetS have elevated hemoglobin, ferritin, erythropoietin and haptoglobin concentrations. Higher hemoglobin levels are related to all components of MetS. Higher ferritin levels associate with TG, abdominal obesity, elevated glucose or low high-density cholesterol. Haptoglobin levels associate with blood pressure or elevated glucose. However, erythropoietin levels are related only with abdominal obesity. Higher serum erythropoietin concentrations may suggest underlying adipose tissue hypoxemia in MetS.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PH wrote the manuscript. JS contributed to discussion and reviewed and edited the manuscript. HK performed the statistical analyses and reviewed and edited the manuscript. PM contributed the discussion and reviewed and edited the manuscript. MV researched data, contributed to discussion and reviewed and edited manuscript. All authors read and approved the final manuscript.

Acknowledgements
Nothing to declare.

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Received: 4 July 2012 Accepted: 22 September 2012
Published: 27 September 2012

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doi:10.1186/1475-2840-11-116
Cite this article as: Hämäläinen *et al.*: Erythropoietin, ferritin, haptoglobin, hemoglobin and transferrin receptor in metabolic syndrome: a case control study. *Cardiovascular Diabetology* 2012 **11**:116.

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Serum ferritin levels and the development of metabolic syndrome and its components: a 6.5-year follow-up study.

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Diabetol Metab Syndr. Oct 26;6(1):114, 2014. doi: 10.1186/1758-5996-6-114.

DIABETOLOGY & METABOLIC SYNDROME

RESEARCH Open Access

Serum ferritin levels and the development of metabolic syndrome and its components: a 6.5-year follow-up study

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Abstract

Background: The aim of this study was to investigate the relationship between changes in serum ferritin concentrations and the development of metabolic syndrome (MetS) and its components over a 6.5 year follow-up period in Finnish adults.

Methods: Adults born in Pieksämäki, Finland, in 1942, 1947, 1952, 1957, and 1962 (n = 1294) were invited to health checkups between 1997 and 1998 and 2003 and 2004. All of the required variables for both checkups were available from 691 (53%) subjects (289 men and 402 women). MetS was defined by the National Cholesterol Education Program criteria.

Results: During the 6.5-year follow-up period, 122 (18%) subjects developed incident cases of MetS. Increases in serum ferritin levels were significantly higher in both women and men with incident MetS compared with women and men without MetS ($p = 0.04$, $p = 0.03$). Also, serum ferritin levels increased significantly less in women in whom the criteria for MetS resolved during the follow-up period ($p = 0.01$). Increases in serum ferritin levels were significantly lower in women in whom the glucose criterion for MetS resolved, and higher in women for whom the waist criterion developed ($p = 0.01$ and $p < 0.001$, respectively). Serum ferritin levels decreased significantly more in men in whom the triglyceride criterion for MetS resolved during the follow-up period ($p = 0.01$). There was a clear and significant correlation between change in serum ferritin level and change in waist circumference both in men and women ($p < 0.001$, $p < 0.01$). In addition, correlations between change in serum ferritin level and change in plasma triglyceride as well as glucose levels were strongly positive in men ($p < 0.001$). There was negative correlation between change in serum ferritin and plasma high density cholesterol level both in men and women.

Conclusions: Increases in serum ferritin over a 6,5 year period are associated with development of MetS in both men and women. Whereas, lower increases in serum ferritin over the same timeframe are associated with resolution of hypertriglyceridemia in men and hyperglycemia in women. Increases in waist circumference was positively correlated with increases in serum ferritin in both men and women.

Keywords: Metabolic syndrome, Ferritin, Obesity

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Background

Metabolic syndrome (MetS) is a pathophysiological disorder with a clustering of risk factors for cardiovascular disease and type 2 diabetes [1,2]. Ferritin, an intracellular protein and key regulator of iron homeostasis, is a clinical measure of body iron stores [3]. Elevated body iron stores could promote oxidative stress, and in this manner affect the pathogenesis of insulin resistance [4-6]. Several studies have reported an association between elevated serum ferritin levels and elevated serum insulin, fasting glucose, insulin resistance [7-9], and diabetes [10-16]. Cross-sectional studies have found an association between metabolic syndrome (MetS) and serum ferritin levels [17-23]. No studies have been done to investigate the relationships between changes in serum ferritin levels and development of MetS components in both men and women. One prospective study previously evaluated an association between baseline serum ferritin levels and future MetS [24].

The aim of this population-based study was to investigate the relationship between changes in serum ferritin over a 6.5 year period and the development and resolution of MetS and its components in Finnish adults.

Methods

All residents of Pieksämäki, a town in Finland, who were born in 1942, 1947, 1952, 1957, and 1962 (n = 1,294) were invited to receive a health checkup between 1997 and 1998 (baseline visit) and again between 2003 and 2004. Of those invited, 923 (71%) participated in the first checkup, and 693 subjects (54%) attended both checkups. All variables analyzed in the present study were available from 691 subjects (289 men and 402 women). All the subjects completed a questionnaire that asked about their medications, smoking habits, alcohol consumption, and level of physical activity. The protocol was approved by the Kuopio University Hospital Ethics Committee. All participants provided written informed consent.

Clinical and laboratory measurements

Health evaluations were performed by the same 2 nurses at both checkups. Sitting blood pressure was measured with a mercury sphygmomanometer after 15 minutes of rest. The measurement was repeated 5 minutes later, and the mean of the 2 measurements was used in the statistical analyses. Waist circumference was measured from the midpoint between the lateral iliac crest and the lowest rib to the nearest 0.5 cm. Weight and height were measured to the nearest 0.1 kg and 0.5 cm, respectively.

Blood samples were taken after an overnight fast. Plasma was separated by centrifugation and the samples were frozen immediately and stored at -70C. Samples were analyzed in Kuopio regional laboratory during the year 2010.

Plasma glucose concentration was measured using an automated colorimetric method (Peridochrom Glucose GOD-PAP; Boehringer Mannheim GmbH, Mannheim, Germany). Serum triglycerides and cholesterol were measured from fresh serum samples using glycerol-3-phosphate oxidase phenol + aminophenazone (PAP) and cholesterol oxidase-PAP (CHOD-PAP) enzymatic colorimetric methods, respectively (Boehringer Mannheim GmbH). Serum high-density lipoprotein (HDL) cholesterol was measured using the same method (CHOD-PAP) after the precipitation of apolipoprotein B-containing lipoprotein particles by phosphotungstic acid and magnesium. High-sensitivity C-reactive protein (hs-CRP) was measured with an Immulite® analyzer and a DPC high-sensitivity CRP assay (Diagnostics Products Corporation, Los Angeles, CA, USA). Serum ferritin concentration was analyzed using an electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). In order to exclude liver storage disease plasma alanine aminotransferase (P-ALT) was measured using a kinetic method according to the International Federation of Clinical Chemistry and Laboratory Medicine using a cobas® 6000 (c 501) analyzer (Hitachi High Technology Co, Tokyo, Japan).

At the beginning and end of the study period, MetS was defined according to the new harmonized criteria [23]. Subjects with 3 or more of the following components were classified as having metabolic syndrome: 1) waist circumference ≥102 cm for men and ≥88 cm for women 2) fasting triglycerides ≥1.7 mmol/L or treatment for dyslipidemia 3) serum HDL cholester <1.03 mmol/L for men and <1.29 mmol/L for women or treatment for dyslipidemia 4) systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg or the use of antihypertensive medication 5) fasting plasma glucose of ≥5.6 mmol/L or the use of medication for hyperglycemia.

Statistical analyses

The results are expressed as means and standard deviations (SDs) for continuous variables and as proportions for categorical variables. The normality of variables was evaluated by the Shapiro-Wilk W-test. Statistical comparisons between the groups were performed using the chi-square test, t-test, or bootstrap-type t-test as appropriate. Bootstrap type analysis of covariance was also used to compare the groups as measurements. In these analyses, the baseline variables of age, smoking, physical activity, serum ferritin levels, body mass index and hs-CRP were used as covariates. Partial correlations were calculated between chance in serum ferritin level and chance in levels of MetS components and adjusted for age, and baseline smoking, physical activity, alcohol use, serum ferritin concentration, body mass index, and hs-CRP. For all analyses, $p < 0.05$ was considered significant.

Results

All variables from both checkups were available for 691 subjects (289 men and 402 women). The baseline characteristics of the study participants are presented in Table 1. At baseline, MetS was present in 31% of the subjects. During the follow-up time, 122 (18%) incident cases of MetS developed and 44 (6%) cases of MetS resolved (data not shown).

Development of MetS and MetS components in relation to changes in serum ferritin levels during the follow-up period are shown in Figure 1. All results were adjusted for the baseline variables of age, smoking, physical activity, use of alcohol, serum ferritin level, body mass index and hs-CRP. Serum ferritin level was significantly higher both in women and men with incident MetS compared with women and men without MetS (p = 0.04, p = 0.03). Serum ferritin levels increased significantly less in women in whom the criteria for MetS resolved during the follow-up period compared with women in whom the MetS criteria remained (p = 0.01). Increases in serum ferritin levels were significantly lower in women in whom the glucose criterion of MetS resolved during the follow-up period compared with women in whom the glucose criterion remained (p = 0.01). Serum ferritin levels decreased significantly more in mean whom the MetS triglyceride criteria for triglycerides resolved during the follow-up period compared to men who continued to meet the MetS criteria for triglycerides (p = 0.004). Also, there was a statistical trend suggesting an increase in serum ferritin over the follow-up period was associated with the development of hypertriglyceridemia compared to men who did not develop hypertriglyceridemia (p = 0.05). Increases in serum ferritin were significantly associated with increases in waist circumference in women during the follow-up period (p = 0.0001). Changes in ferritin levels between subjects whose HDL or blood pressure criterion developed or resolved during follow-up time did not differ significantly.

Partial correlations between change in serum ferritin level and change in levels of MetS components are shown in Table 2. All results are adjusted for the baseline variables of age, smoking, physical activity, use of alcohol, serum ferritin level, body mass index and hs-CRP. There was strong positive correlation between change in serum ferritin level and change in waist circumference both in men and women (p <0.001, p <0.01). In addition,

Table 1 Baseline clinical and life-style characteristics of the study population

Characteristics	Men N = 289 (42%)	Women N = 402 (58%)	All N = 691
MetS criteria present, n (%)	98 (34)	116 (29)	214 (31)
Age, years, mean (SD)	45.3 (6.2)	45.1 (6.5)	45.2 (6.2)
BMI, mean (SD)	26.8 (3.5)	26.3 (5.2)	26.6 (4.6)
Waist, cm, mean (SD)	94.2 (10.2)	83.4 (12.3)	87.9 (12.6)
FP-glucose mmol/L, mean (SD)	5.9 (0.9)	5.6 (0.6)	5.8 (0.8)
BP systolic, mmHg, mean (SD)	137.7 (16.5)	132.2 (18.2)	134.4 (0.7)
BP diastolic, mmHg, mean (SD)	83.5 (9.7)	79.4 (9.5)	81.1 (9.8)
HDL-C, mmol/L, mean (SD)	1.3 (0.3)	1.5 (0.3)	1.4 (0.3)
Triglycerides, mmol/L, mean (SD)	1.7 (1.3)	1.2 (0.6)	1.4 (1.0)
ALT (U/L) mean (SD)	18.0 (10.9)	12.0 (7.6)	14.5 (9.6)
Hs-CRP (mg/L), mean (SD)	1.7 (3.9)	1.7 (2.3)	1.7 (3.0)
Life-style factors, n (%)			
Current smoker	80 (28)	82 (20)	162 (23)
Current use of alcohol			
Low (nothing)	36 (12)	85 (21)	121 (18)
Moderate	167 (58)	290 (72)	457 (66)
High	86 (30)	27 (7)	113 (16)
Physical activity n (%):			
Low	41 (14)	54 (13)	95 (14)
Moderate	161 (56)	240 (60)	401 (58)
High	87 (30)	108 (27)	195 (28)

BMI: Body mass index; FP-glucose: fasting plasma glucose; BP systolic: systolic blood pressure; BP diastolic: diastolic blood pressure; HDL-C: high density cholesterol; Hs-CRP: high sensitivity C-reactive protein; ALT: alanine aminotransferase.
Current use of alcohol was considered low with no use of alcohol, moderate with use of <2 portions/day, and high with use of >2 portions/day. Physical activity was considered to be high in subjects who exercised at least 30 minutes daily in their leisure time, moderate in subjects who exercised at least three times per week, and low if exercising frequency was less than three times per week.

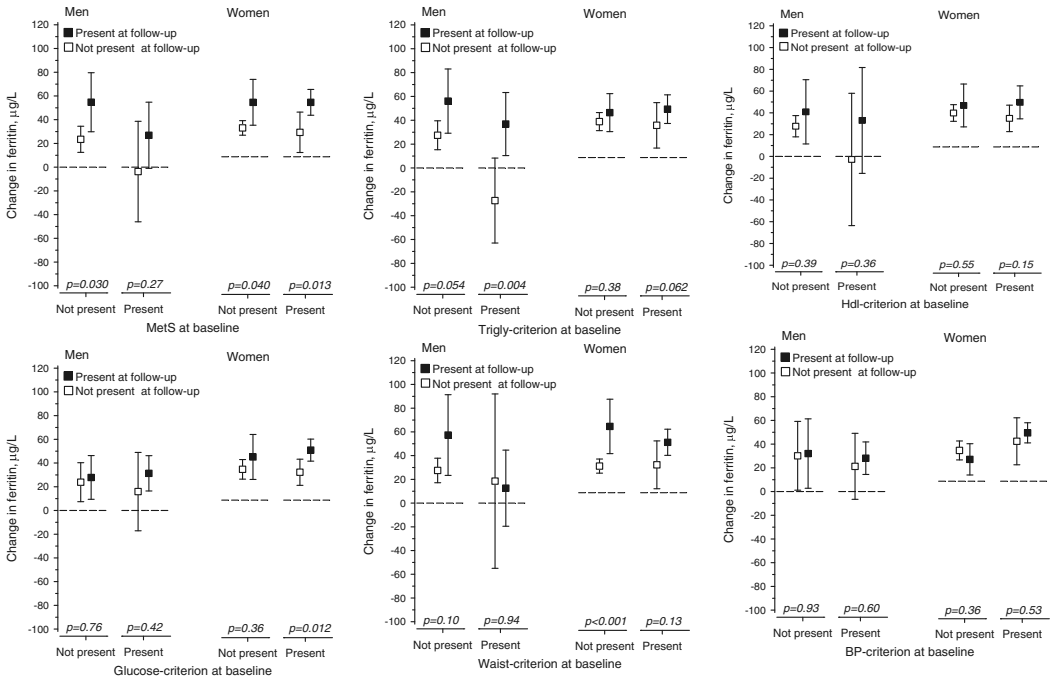

Figure 1 Legends: All results are adjusted for age, baseline smoking, baseline physical activity, baseline use of alcohol, baseline serum ferritin level, baseline body mass index and baseline hs-CRP. Waist criterion = Waist >102 cm (male) or >88 cm (female), Glucose criterion = FP-glucose ≥5.6 mmol/L, Trigly criterion = Triglycerides >1.7 mmol/l or medication for dyslipidemia, HDL criterion = HDL–cholesterol <1.03 mmol/l (men) or <1.29 mmol/l (women) or medication for dyslipidemia, BP criterion = Systolic blood pressure ≥130 mmHg or diastolic ≥85 mmHg or antihypertensive medication.

correlations between change in serum ferritin level and change in plasma triglyceride as well as glucose levels were strongly positive in men (p <0.001). There was negative correlation between change in serum ferritin and change in plasma HDL cholesterol level both in men and women. The correlations between change in serum ferritin level and change in systolic or diastolic blood pressure level were not significant.

Table 2 Partial correlations between change in serum ferritin level and change in levels of MetS components

	Men r (95% CI)	Women r (95% CI)
Waist	0.21 (0.11 to 0.35)***	0.15 (0.05 to 0.24)**
Triglycerides	0.20 (0.11 to 0.31)***	0.09 (-0.01 to 0.17)
HDL-C	-0.12 (-0.25 to -0.01)*	-0.13 (-0.22 to -0.02)*
Glucose	0.20 (0.07 to 0.33)***	0.08 (-0.02 to 0.18)
Systolic BP	-0.03 (-0.15 to 0.11)	0.02 (-0.07 to 0.12)
Diastolic BP	0.03 (-0.11 to 0.18)	0.03 (-0.09 to 0.13)

*p <0.05, **p <0.01, ***p <0.001.
Adjusted for age, baseline smoking, baseline physical activity, baseline use of alcohol, baseline serum ferritin level, baseline body mass index and baseline high sensitivity C-reactive protein.
HDL: high density cholesterol; Systolic BP: systolic blood pressure; Diastolic BP: diastolic blood pressure.

Discussion

To the best of our knowledge, this is the first follow-up study evaluating changes in serum ferritin levels in relationship with the development and resolution of MetS and components of the MetS in Finnish men and women.

The increase in serum ferritin levels during the follow-up period was significantly higher in men and in women with incident MetS compared with men and women without incident MetS. In addition, reductions in ferritin levels were significantly higher in women in whom the criteria of MetS resolved during the follow-up period. Previous longitudinal studies have evaluated changes in ferritin levels and MetS [17,18,20,21,23,25-27]. Also, a recent longitudinal study showed that elevated ferritin levels at baseline are associated with future development of MetS in Korean men [24]. Our study shows not only an association between baseline increased ferritin levels and future MetS, but also between increased or reduced

ferritin levels and MetS development or resolution, respectively, during the follow-up period. We also show an association in both sexes and extended our evaluation to all MetS components. No previous longitudinal studies have been done to evaluate the changes in ferritin levels and development of all MetS components.

We show that serum ferritin was significantly higher or trended towards being higher in men with the triglyceride criterion for MetS resolved or developed over the follow-up period. We also show strong positive association between change in serum ferritin level and triglyceride level in men. This agrees with previous cross-sectional studies that found an increasing prevalence of elevated triglycerides with increasing serum ferritin levels [20,26]. Other studies showed that higher ferritin concentrations were associated with increased triglyceride concentrations [23,27]. Also, higher ferritin concentrations were previously found to be associated with an increase in triglyceride levels in male patients with iron overload and homozygosity for human hemochromatosis gene mutations [28].

We show that serum ferritin levels increased significantly less in women in whom the glucose criterion for MetS resolved during the follow up period and also a strong positive association between change in serum ferritin level and glucose level in men. Several longitudinal studies have previously shown an association between incident diabetes and higher baseline ferritin levels [10,12-16,29].

Serum ferritin levels increased significantly more in women whose waist criterion for MetS developed during the follow up period. Also, there was a strong positive correlation between change in serum ferritin level and change in waist circumference both in men and women. Waist circumference was the only one of the Mets components that was positively associated with change in ferritin level both in men and women, which may indicate the importance of waist circumference in development of Mets. Our results are in line with previous studies that found an association between serum ferritin levels and central adiposity [30] or an increasing prevalence of the MetS waist criterion with increasing serum ferritin levels [23,26]. However, parts of the previous cross-sectional studies found no association between the MetS waist criterion and ferritin levels [27].

The mechanism underlying serum ferritin levels and the development of MetS is not established, but iron accumulation and oxidative stress is the leading hypothesis. Iron is involved in multiple cellular processes and is important for the activity of various enzymes, but it can also be toxic and cause organic biomolecular oxidation [31]. Ferritin is a clinical measure of body iron stores [3]. Elevated body iron stores may promote oxidative stress, that may contribute to cellular damage leading to insulin dysfunction, insulin resistance and abnormal pancreatic beta-cell function [2,4-6,32]. Hepatic iron overload has been shown to contribute to peripheral hyperinsulinemia and insulin resistance, while muscular iron accumulation contributes to decreased glucose utilization [33]. Moreover, phlebotomy with a moderate reduction in body iron stores measured by serum ferritin levels resulted in improvements in glycemic control in patients with MetS in a controlled clinical trial [34]. Chronic oxidative stress is also associated with oxidation dysfunction of long chain fatty acids in mitochondria, which can lead to hypertriglyceridemia in circulation and excessive triglyceride accumulation in muscle and liver tissue [35,36]. These mechanisms support our findings that increasing ferritin levels predict incident MetS and hyperglycemia and hypertriglyceridemia components of MetS.

The iron-regulatory hormone hepcidin control the dietary absorption, storage, and tissue distribution of iron. When hepcidin concentrations are high, iron is trapped in enterocytes, macrophages, and hepatocytes [37]. Adipose tissue expressed hepcidin has shown to be enhanced in obese patients [38]. It is possible, that when waist circumference and central adiposity increase, also hepcidin expression increases, and altered iron homeostasis leads to a change in ferritin levels.

Because ferritin is also an acute phase reactant, all results in our study were adjusted for hs-CRP levels to estimate the impact of inflammation. Results remained significant after adjustment that can suggest that ferritin concentrations are reflective of storage iron and not the acute phase response.

Some limitations of our study should be mentioned. We were not able to exclude persons with known *HFE* gene mutations. However, there was no need to exclude subjects with high baseline serum ferritin levels, considering that all baseline ferritin levels were less than 750 μg/l (range, 2–722 μg/l). Also, all baseline plasma ALT levels were less than 120 U/L (range, 4-120 U/L) that does not refer liver storage disease. Unfortunately, information about the female participant's menopausal status or possible change in status during the follow up period was not available. Mean ferritin levels are known to be higher in premenopausal women than in postmenopausal ones [26,39]. Also, information about the nutritional content of subjects' diets or their consumption of dietary supplements like iron or antioxidants were not available. The relatively small number of MetS cases that resolved during the follow up period is also a limitation, and could have affected the non-significant results observed in men. However, the longitudinal, population-based design is the strength of this study.

In conclusion, serum ferritin level works as a follow-up and risk assessment measure in subjects with

metabolic risk factors; increasing levels indicating developing MetS and decreasing levels indicating resolving hypertriglyceridemia and hyperglycemia.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PH wrote the manuscript; JS contributed to the discussion and reviewed the manuscript; HK researched the data, contributed to the discussion, and reviewed the manuscript; MV researched the data, contributed to the discussion, and reviewed the manuscript; PM contributed to the discussion, and reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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Received: 1 April 2014 Accepted: 14 October 2014
Published: 26 October 2014

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doi:10.1186/1758-5996-6-114
Cite this article as: Hämäläinen *et al.*: Serum ferritin levels and the development of metabolic syndrome and its components: a 6.5-year follow-up study. *Diabetology & Metabolic Syndrome* 2014 **6**:114.

III

Hemoglobin level and lipoprotein particle size.

Hämäläinen P, Saltevo J, Kautiainen H, Mäntyselkä P and Vanhala M.

Lipids Health Dis. Jan 10;17(1):10, 2018. doi: 10.1186/s12944-018-0655-2.

Hämäläinen et al. Lipids in Health and Disease (2018) 17:10
DOI 10.1186/s12944-018-0655-2

Lipids in Health and Disease

RESEARCH Open Access

Hemoglobin level and lipoprotein particle size

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Abstract

Background: Alterations in lipoprotein size are associated with increased cardiovascular disease risk. Higher hemoglobin levels may indicate a higher risk of atherosclerosis and was previously associated with obesity, metabolic syndrome, and insulin resistance. No previous studies have investigated an association between hemoglobin concentration and lipoprotein particle size.

Methods: We conducted a population-based, cross-sectional study of 766 Caucasian, middle-aged subjects (341 men and 425 women) born in Pieksämäki, Finland, who were categorized into five age groups. The concentrations and sizes of lipoprotein subclass particles were analyzed by high-throughput nuclear magnetic resonance (NMR) spectroscopy.

Results: Larger very low density lipoprotein (VLDL) particle diameter was associated with higher hemoglobin concentrations in men ($p = 0.003$). There was a strong relationship between smaller high density lipoprotein (HDL) particle size and higher hemoglobin concentration in both men and women as well as with smaller low density lipoprotein (LDL) particle size and higher hemoglobin concentration in men and women ($p < 0.001$; $p = 0.009$, $p = 0.008$). VLDL particle concentration had a moderate positive correlation with hemoglobin concentration ($r = 0.15$; $p < 0.001$). LDL particle concentration showed a statistical trend suggesting increasing particle concentration with increasing hemoglobin levels ($r = 0.08$; $p = 0.05$).

Conclusion: Higher hemoglobin levels are associated with larger VLDL, smaller LDL, and smaller HDL particle sizes and increasing amounts of larger VLDL and smaller LDL particles. This suggests that a higher hemoglobin concentration is associated with an unfavorable lipoprotein particle profile that is part of states that increase cardiovascular disease risk like diabetes and metabolic syndrome.

Keywords: Liporotein particle size, VLDL, LDL, HDL, Hemoglobin

Background

Lipoproteins consist of heterogeneous particles that differ in size. Alterations in lipoprotein sizes are associated with increased cardiovascular disease (CVD) risk [1–4] as well as in patients with conditions in which CVD risk is high like diabetes and metabolic syndrome [5–9]. Higher hemoglobin levels may indicate a higher risk of atherosclerosis [10] and was previously associated with obesity, metabolic syndrome, and insulin resistance [10–16]. However, as far as we are aware, no previous studies have investigated an association between hemoglobin concentration and lipoprotein particle size. Therefore, we conducted a cross-sectional study investigating any association between hemoglobin level and lipoprotein particle size assessed by proton nuclear magnetic resonance (NMR) spectroscopy.

Methods

Study subjects

The study population primarily consisted of 1294 middle-aged subjects from Pieksämäki, Finland, who were born in 1942, 1947, 1952, or 1962. These subjects were invited to a health check-up in the years 1997–1998 initially, and to a follow-up check-up in 2003–2004. A total of 766 subjects participated in the second health check-up in 2003–2004 when the hematological laboratory tests were

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performed. The final analysis included data from these 766 subjects. The study protocol was approved by the Ethics Committee of Kuopio University Hospital and the University of Eastern Finland. All participants provided informed written consent.

Clinical and laboratory procedures

Both health check-ups were performed by the same two nurses. Waist circumference was measured and body mass index (BMI) was calculated. Current use of alcohol was considered low if the subject used no alcohol, moderate if the subject used less than two units per day, and high if the subject used more than two units per day. Physical activity was considered low if the subject exercised less than 30 min fewer than 3 times per week, moderate if the subject exercised at least 3 times per week, and high if the subject exercised every day.

Fresh blood samples were taken after an overnight fast. Plasma glucose concentration was measured using an automated colorimetric method (Peridochrom Glucose GOD-PAP, Boehringer, Germany). Plasma triglycerides were measured from fresh serum samples using enzymatic colorimetric methods (CHOD-PAP, GPO-PAP, Boehringer Mannheim GmbH, Germany). Plasma high density lipoprotein (HDL) cholesterol was measured using the same method after precipitation of low-density lipoprotein (LDL) cholesterol and very low-density lipoprotein (VLDL) cholesterol with phosphotungstic acid and magnesium. High-sensitivity C-reactive protein (hs-CRP) was measured with an Immunolite analyzer and a DPC hs-CRP assay (DPL, Los Angeles, CA, USA). Hemoglobin was measured using an automatic electronic cell calculator. All laboratory tests were analyzed at the Kuopio University laboratory during the years 2009–2010.

Concentrations and sizes of lipoprotein subclass particles were analyzed with high-throughput NMR spectroscopy of native serum samples [17, 18] in 2009. NMR data were measured at 37 °C using a Bruker AVANCE III spectrometer operating at 500.36 MHz using a new automated platform, as described previously [19]. The following 14 lipoprotein subclasses were calibrated using high-performance liquid chromatography: chylomicrons (CMs) and largest VLDL particles (CM/ largest VLDL; average particle diameter ± 75 nm); five different VLDL subclasses, i.e., very large (average particle diameter 64.0 nm), large (53.6 nm), medium (44.5 nm), small (36.8 nm) and very small VLDL (31.3 nm); intermediate-density lipoprotein (IDL; 28.6 nm); three LDL subclasses, i.e., large (25.5 nm), medium (23.0 nm), and small LDL (18.7 nm); and four HDL subclasses, i.e., very large (14.3 nm), large (12.1 nm), medium (10.9 nm), and small HDL (8.7 nm).

Statistical methods

The data are presented as means and standard deviations. The 95% confidence intervals for the lipoprotein particle concentrations (and diameters) were obtained by bias-corrected, accelerated bootstrapping. Associations between the serum triglyceride, HDL, and total cholesterol concentrations with the NMR-measured concentrations were estimated with regression analysis using Sidak-adjusted probabilities. Multiple linear regression analysis was used to estimate the independent impacts of LDL, HDL, and VLDL particle diameter on the hemoglobin stratified by sex. In all hypotheses, $p < 0.05$ was considered significant.

Results

Basic charasteristics of the study population are shown in Table 1. The study population included 425 women

Table 1 Clinical and life-style characteristics of the study population

Characteristics	Men $N = 341$	Women $N = 425$	All $N = 766$
Age, years, mean (SD)	52.5 (6.2)	52.2 (6.5)	52.4 (6.4)
BMI, kg/m2, mean (SD)[a]	27.3 (3.9)	27.1 (5.0)	27.2 (4.6)
Waist, cm, mean (SD)	96.3 (11.3)	86.6 (12.2)	90.6 (12.8)
FP-glucose (mmol/L), mean (SD)[b]	6.1 (1.1)	5.8 (0.8)	5.9 (0.9)
Total cholesterol (mmol/L), mean (SD)	5.6 (0.9)	5.4 (1.0)	5.5 (1.0)
HDL-C (mmol/L), mean (SD)[c]	1.5 (0.4)	1.7 (0.3)	1.6 (0.3)
Triglycerides (mmol/L), mean (SD)	1.5 (1.0)	1.2 (0.5)	1.3 (0.7)
Hemoglobin, (g/L), mean (SD)	152.8 (9.2)	137.8 (9.0)	144.0 (11.7)
Hs-CRP (mg/L), mean (SD)[d]	1.8 (3.2)	2.1 (2.8)	2.0 (2.9)
Alat (l/U), mean (SD)	18.0 (10.9)	12.0 (7.6)	14.5 (9.6)
Creatinine (μmol/L), mean (SD)	87.1 (10.2)	75.8 (7.5)	80.1 (8.7)
Life-style factors, n (%)			
Current smoker	88 (26)	74 (17)	162 (21)
Current use of alcohol[e]			
Low (nothing)	46 (13)	92 (22)	138 (18)
Moderate	165 (49)	274 (64)	439 (57)
High	129 (38)	55 (13)	184 (24)
Physical activity n (%):			
Low	89 (26)	135 (32)	224 (29)
Moderate	193 (57)	238 (56)	431 (56)
High	59 (17)	49 (12)	108 (14)

Physical activity: Low = at least 30 min exercise less than 3 times/week, Moderate = at least 30 min exercise at least 3 times/week, High: e at least 30 min exercise daily

[a]BMI: Body mass index

[b]FP-glucose: fasting plasma glucose

[c]HDL-C: high density cholesterol

[d]Hs-CRP: high sensitivity C-reactive protein

[e]Current use of alcohol: Low = Nothing, Moderate = <2portions/day, High= >2 portions/day

(55%) and 341 men with a mean age of 52.4 years and a mean body mass index (BMI) 27 kg/m^2. Kidney function based on plasma creatinine level both in men and women was normal. Plasma alanine aminotransferase (alat) level was in normal female reference range (10–45 IU/L) in 99.5% of the women and in normal male reference range (10–70 IU/l) in 99.0% of men. Current smokers were 26% of men and 17% of women. Correlations between fasting plasma triglycerides, HDL, total cholesterol, and LDL, HDL and VLDL particle concentrations measured by NMR in women and men are shown in Table 2. Total plasma cholesterol had a high positive correlation with NMR-measured LDL particle concentration in both women and men (r = 0.92 and r = 0.89, respectively; $p < 0.001$). Also, plasma total cholesterol had a moderate positive correlation with NMR-measured VLDL concentration in women and men (r = 0.50 and r = 0.43, respectively; $p < 0.001$). Plasma triglycerides had a high positive correlation with the NMR-measured VLDL particle concentration in women (r = 0.85; $p < 0.001$) and a moderate positive correlation in men (r = 0.58; $p < 0.001$). Plasma HDL cholesterol had a high positive correlation with the NMR-measured HDL particle concentration in both women and men (r = 0.67 and r = 0.70, respectively; $p < 0.001$). There was no significant correlation between plasma triglycerides, HDL, or total cholesterol and NMR-measured LDL, HDL, or VLDL particle diameter (*Data not shown*).

Figure 1 shows standardized coefficients (beta) between lipoprotein particle diameters and hemoglobin level. All values were first (Fig. 1a) adjusted for age, hs-CRP, and NMR-measured LDL, HDL, or VLDL concentrations. Larger VLDL particle diameter was associated with higher hemoglobin concentrations in both men and women (p = 0.002 and p = 0.029, respectively). There was a strong relationship between smaller HDL particle size and higher hemoglobin concentration in both men and women as well as lower LDL particle size and higher hemoglobin concentration in men ($p < 0.001$). Also, lower LDL particle size was associated with higher hemoglobin concentrations in women (p = 0.002). After adding adjusted-model the homeostasis model for assessment of insulin resistance (HOMA-IR) (Fig. 2b), all results remain significant except larger VLDL diameter in women (p = 0.073). No significant association was found between serum ferritin level and the lipoproteins VLDL, LDL, or HDL particle diameter (data not shown).

Correlations between hemoglobin concentration and NMR-measured VLDL, LDL, and HDL particle concentration are shown in Fig. 2a-c. VLDL particle concentration had a moderate positive correlation with hemoglobin concentration (r = 0.15; $p < 0.001$). LDL particle concentration showed a statistical trend suggesting increasing particle concentration with increasing hemoglobin levels (r = 0.08; p = 0.05). There was no significant correlation between HDL particle concentration and hemoglobin.

Table 2 Correlations between plasma triglycerides, HDL or total cholesterol and NMR-measured LDL, HDL or VLDL particle concentration

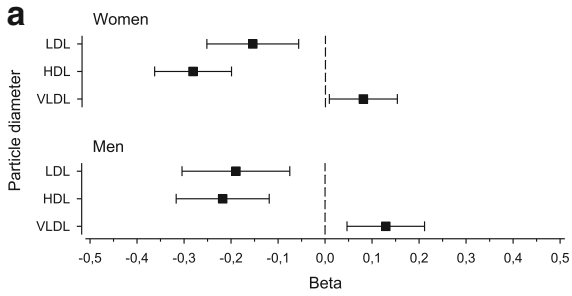
Plasma cholesterol	NMR-measured particle concentrations		
	LDL r (95% CI)	HDL r (95% CI)	VLDL r (95% CI)
Women			
Triglycerides	0.29[a]	−0.07	0.85[a]
HDL	0.09	0.67[a]	−0.37[a]
Total	0.92[a]	0.25[a]	0.50[a]
Men			
Triglycerides	−0.01	−0.03	0.58[a]
HDL	0.16[b]	0.70[a]	−0.45[a]
Total	0.89[a]	0.34[a]	0.43[a]

Sidak-adjusted probabilities
[a] $p < 0.001$
[b] $p < 0.05$

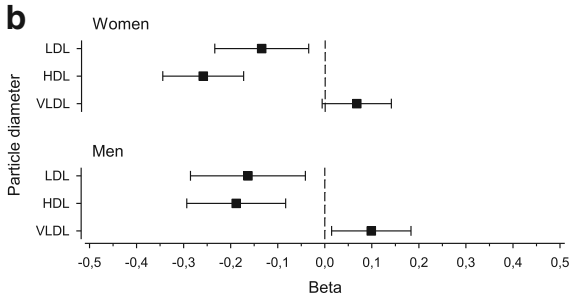
Discussion

In present study, we show an association between the particle size of the lipoproteins VLDL, HDL, and LDL and hemoglobin level. Although, associations between lipoprotein particle size and CVD, metabolic syndrome, obesity, insulin resistance, and type 2 diabetes have been investigated, we are not aware of previous studies reporting an association between hemoglobin concentration and lipoprotein particle size.

Larger VLDL particle size, smaller LDL particle size, and smaller HDL particle size were associated with higher hemoglobin concentrations. These associations remained unchanged after adjusting for concentrations of VLDL, LDL, or HDL particles. Previously, larger mean VLDL particle size was associated with impaired glucose tolerance, insulin resistance, and incidence of type 2 diabetes [5–9]. Also, previous studies showed an association between small LDL particle size and insulin resistance as well as incident diabetes, although the association with diabetes was not independent after adjusting for insulin sensitivity or triglycerides [8]. Additionally, small HDL particles were previously associated with reduced insulin sensitivity and hyperglycemia [7]. Our findings suggest that higher hemoglobin concentrations are associated with an unfavorable lipoprotein particle profile that is part of conditions such as metabolic syndrome and type 2 diabetes that increase CVD risk. Consequently, higher



Beta = Hemoglobin standardized coefficients
Women: LDL particle size p=0.002, HDL particle size p<0.001, VLDL particle size p=0.029
Men: LDL particle size p<0.001, HDL particle size p<0.001, VLDL particle size p=0.002
All values are adjusted for age, hs-CRP and LDL, HDL or VLDL NMR-measured particle concentration



Beta = Hemoglobin standardized coefficients
Women: LDL particle size p=0.008, HDL particle size p<0.001, VLDL particle size p=0.073
Men: LDL particle size p=0.009, HDL particle size p<0.001, VLDL particle size p=0.003
All values are adjusted for age, hs-CRP, HOMA-IR, and LDL, HDL or VLDL NMR-measured particle concentration

Fig. 1 LDL, HDL and VLDL particle diameter changes in relation to hemoglobin concentration in women and men. **a** Beta = Hemoglobin standardized coefficients. Women: LDL particle size $p = 0.002$, HDL particle size $p < 0.001$, VLDL particle size $p = 0.029$. Men: LDL particle size $p < 0.001$, HDL particle size $p < 0.001$, VLDL particle size $p = 0.002$. All values are adjusted for age, hs-CRP and LDL, HDL or VLDL NMR-measured particle concentration. **b** Beta = Hemoglobin standardized coefficients. Women: LDL particle size $p = 0.002$, HDL particle size $p < 0.001$, VLDL particle size $p = 0.029$. Men: LDL particle size $p < 0.001$, HDL particle size $p < 0.001$, VLDL particle size $p = 0.002$. All values are adjusted for age, hs-CRP, HOMA-IR and LDL, HDL or VLDL NMR-measured particle concentration

hemoglobin concentration can act as an additional marker indicating higher CVD risk profile.

Increasing VLDL particle concentration as well as increasing LDL particle concentration was associated with higher hemoglobin concentration, although the associations were weaker than with particle sizes. Consequently, higher hemoglobin level is associated with an increasing amount of larger VLDL and smaller LDL particles.

Previously, increasing hemoglobin level was associated with increasing arterial stiffness, which is used to assess CVD in high-risk populations [10]. Also, higher hemoglobin concentrations are present in individuals with metabolic syndrome or insulin resistance versus healthy controls or obese subjects versus non-obese subjects [11–16].

In hematological disorder polysytemia vera, elevated hemoglobin levels are associated with hypocholesterolemia and lower serum levels of total cholesterol and LDL cholesterol compared to subjects with elevated hemoglobin but without polysytemia vera [20]. Although studies investigating the association between elevated hemoglobin level and lipoprotein particle size in polysytemia vera patients (as far as we know) have not been done, hypocholesterolemia and lower LDL in polysytemia vera suggest different relation and mechanism than in our study.

The mechanisms that could explain the association between hemoglobin level and changes in lipoprotein particle size are unclear. Preliminary evidence of a relationship between hyperinsulinemia or insulin resistance and stimulated erythropoiesis exists [21]. Insulin can act as a growth factor for erythroid precursors [22]. Our

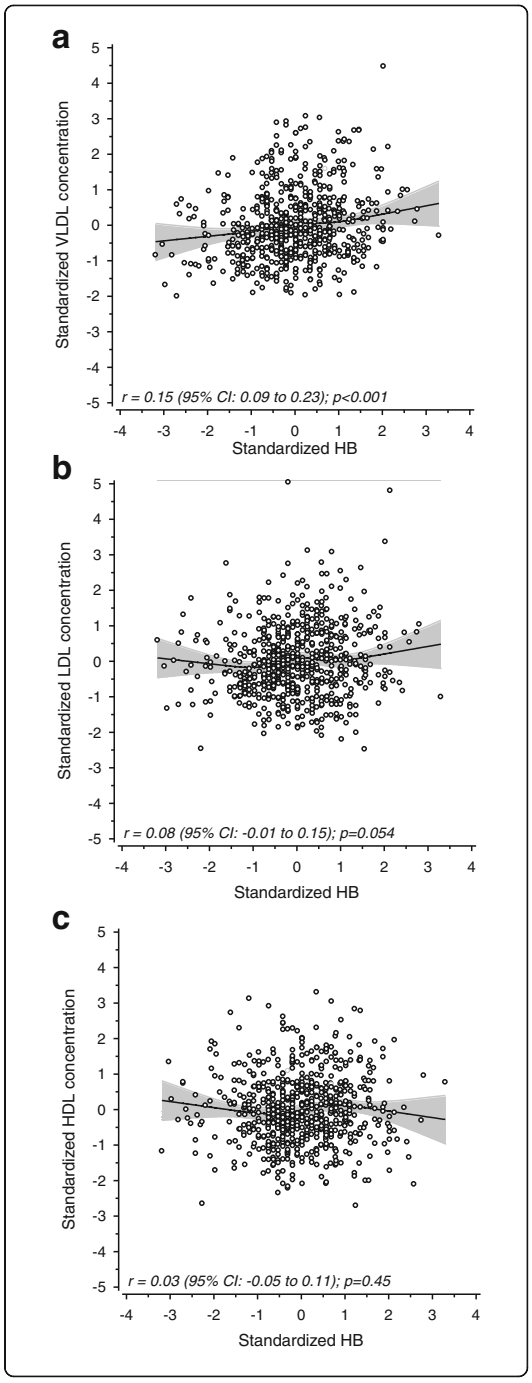


Fig. 2 **a** Correlation between hemoglobin concentration and NMR measured VLDL particle concentration. **b** Correlation between hemoglobin concentration and NMR measured LDL particle concentration. **c** Correlation between hemoglobin concentration and NMR measured HDL particle concentration. All values are adjusted for gender, age and Hs-CRP

results were also adjusted for HOMA-IR to evaluate the influence of insulin resistance. Adjusting did not change the results significance except the association of VLDL particle size and hemoglobin in women. This suggests that insulin resistance mostly affects the VLDL particles, but there exist also other mechanisms affecting the relation of lipoprotein particle size and hemoglobin level.

Hemoglobin level is influenced by iron status. We also investigated an association between serum ferritin level, as an indicator of iron stores, and lipoprotein particle size. Our results suggest that the association between hemoglobin and lipoprotein particle size is independent of serum ferritin levels and iron stores. Also, an association between lipoprotein particle size and hemoglobin level was significant after adjusting for hs-CRP level when excluding the influence of inflammation.

A limitation of our study is the cross-sectional design that does not allow us to identify proper causal relationships. However, the randomly selected, relatively large population, with no exclusion criteria is a strength of this study.

Conclusions

In conclusion, higher hemoglobin levels are associated with larger VLDL, smaller LDL, and smaller HDL particle sizes and increasing amounts of larger VLDL and smaller LDL particles. This suggests that higher hemoglobin concentration is associated with an unfavorable lipoprotein particle profile that is part states that increase CVD risk like diabetes and metabolic syndrome.

Abbreviations

BMI: Body mass index; CMs: Chylomicrons; CVD: Cardiovascular disease; HDL: High density lipoprotein; hs-CRP: High-sensitivity C-reactive protein; LDL: Low density lipoprotein; NMR: High-throughput nuclear magnetic resonance (spectroscopy); VLDL: Very low density lipoprotein

Acknowledgements

None.

Funding

Not applicaple.

Availability of data and materials

Data analyzed during this study is included in this article. All data generated or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
PH, JS, MV: Contributed to the study design, analysis and interpretation of the data and critical revision of the manuscript; PH: Contributed drafting of the manuscript, HK: contributed to the analysis and interpretation of the data and statistical analysis; PM: Contributed to critical revision of the manuscript; and all authors have read and approved the final manuscript.

Ethics approval and consent to participate
The study protocol was approved by the Ethics Committee of Kuopio University Hospital and the University of Eastern Finland. All participants provided informed written consent.

Consent for publication
Not applicable.

Competing interests
All authors declare that they have no competing interests.

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Received: 8 November 2017 Accepted: 2 January 2018
Published online: 10 January 2018

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PÄIVI HÄMÄLÄINEN

Metabolic syndrome (MetS) is a clustering of risk factors that is associated with increase in cardiovascular outcomes, type 2 diabetes and mortality. More information is needed on the factors affecting the progression of MetS as well as markers for clinical medicine for the detection of high risk patients. The aim of this study was to investigate whether the markers of hypoxia, adipose tissue dysfunction and iron metabolism are associated with MetS, its components and MetS progression.

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**PUBLICATIONS OF
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Dissertations in Health Sciences

ISBN 978-952-61-3207-5
ISSN 1798-5706