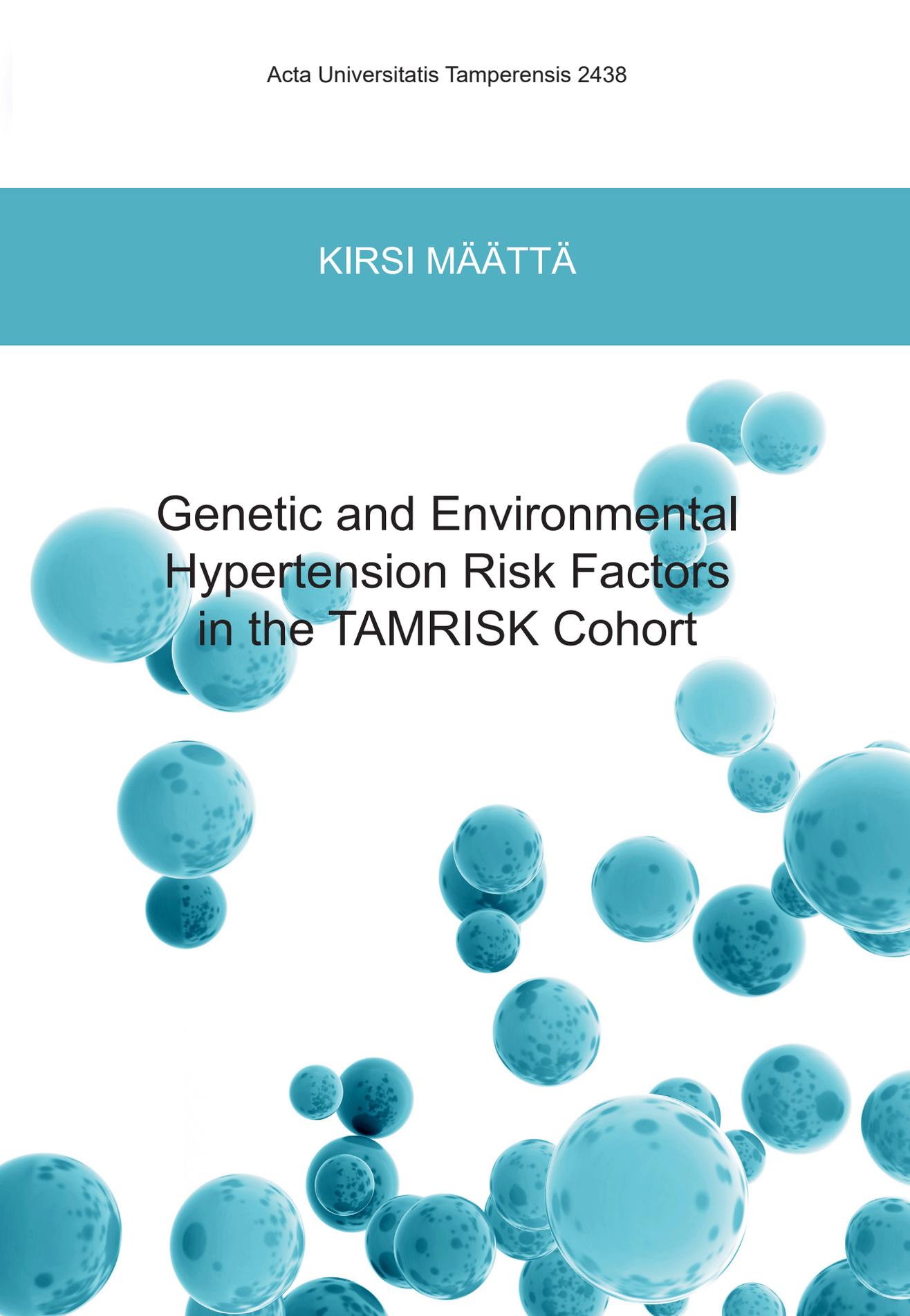


KIRSI MÄÄTTÄ

Genetic and Environmental  
Hypertension Risk Factors  
in the TAMRISK Cohort





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ACADEMIC DISSERTATION

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the Faculty Council of the Faculty of Medicine and Life Sciences  
of the University of Tampere,  
for public discussion in the Jarmo Visakorpi auditorium  
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UNIVERSITY OF TAMPERE

KIRSI MÄÄTTÄ

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## ACADEMIC DISSERTATION

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# ABSTRACT

High blood pressure is a major health problem worldwide. Hypertension is one of the most important risk factors for cardiovascular diseases and it forms a significant burden for healthcare. Blood pressure is affected by genetic and environmental factors such as overweight, salt intake and exercise. Periodic health examinations (PHE) aim in the early diagnosis of disease and they are part of the preventive medicine for chronic disease. Blood pressure rises due to age, but genetic factors may cause hypertension already at young age. This raises the risk for severe consequences at older age even more. Although genetic background of hypertension has been widely studied, majority still remains unfound. Studies suggest a complex polygenic inheritance of hypertension. Heritability of blood pressure is approximated as 30-50 %. There are a lot of candidate genes affecting blood pressure and new gene loci are found continuously. Some new candidate genes for hypertension are here presented. Serine-threonine kinase coding gene *STK39* is a part of the multigene kinase network. It regulates renal Na<sup>+</sup> and K<sup>+</sup> excretion. Also vascular endothelium has a significant role in regulation of blood pressure. Polymorphism in the Solute Carrier Family 7 member 1 (*SLC7A1*) gene changes L-arginine transport and affects endothelial NO production, which can lead to hypertension. *HFE* gene codes for a transmembrane protein acting in the body iron uptake. Polymorphism in *HFE* gene causes a dysfunctional protein and may lead to iron overload. According to new studies, disturbances in iron metabolism may possibly cause hypertension among other severe consequences. Hypertension is a very common disease and antihypertensive therapy is widely used, but the treatment goals are poorly attained. The aims of the present study were to investigate the association of genes *STK39*, *SLC7A1* and *HFE* with hypertension in a Finnish cohort and also investigate the role of periodic cohort health examinations for body mass index and blood pressure during 15 years of follow-up. The main findings of this study were that there was a significant association between rs6749447 in *STK39* and rs1799945 in *HFE* with hypertension, there was no association with rs41318021

in *SLC7A1* and that the effect of PHE was not as efficient as expected on subjects already slightly overweight at baseline.

# TIIVISTELMÄ

Korkea verenpaine on maailmanlaajuisesti hyvin merkittävä terveysongelma. Kohonnut verenpaine on yksi tärkeimmistä riskitekijöistä sydän- ja verenkiertoelimistön sairauksille ja lisäksi se kuormittaa voimakkaasti terveydenhuoltojärjestelmää. Verenpaineeseen vaikuttavat geneettisten tekijöiden lisäksi myös ympäristötekijät kuten ylipaino, suolankäyttö ja liikunnan määrä. Säännöllisillä terveystarkastuksilla tähdätään sairauksien varhaiseen tunnistamiseen ja ne ovat osa kroonisten sairauksien ennaltaehkäisyä. Verenpaine nousee iän myötä, mutta geneettiset tekijät voivat aiheuttaa verenpaineen nousua jo nuorellakin iällä. Tämä lisää vakavien komplikaatioiden riskiä iäkkäämpänä. Korkean verenpaineen geneettistä taustaa on tutkittu paljon, mutta silti merkittävä osa verenpaineen säätelyyn vaikuttavista geneettisistä tekijöistä on vielä löytämättä. Tutkimusten perusteella vaikuttaa siltä, että korkean verenpaineen taustalla on monitekijäinen periytyvyys. Perinnöllisyyden osuuden arvioidaan olevan 30–50 %. Geenitaustaa on tutkittu laajasti ja tutkitaan edelleen, silti tulokset vaihtelevat ja yhden geenin vaikutus on pieni. Tässä tutkimuksessa esitellään kolme uutta verenpaineeseen vaikuttavaa kandidaattigeeniä. Seriini-treoniinikinaasia koodaava geeni *STK39* on osa useiden geenien muodostamaa kinaasiverkostoa, joka säätelee natriumin ja kaliumin eritystä munuaisista. Myös verisuonten endoteelillä on merkittävä rooli verenpaineen säätelyssä. *SLC7A1*-geenin polymorfismi vaikuttaa L-arginiinin kuljetukseen ja endoteelin NO-tuotantoon, mikä puolestaan voi johtaa verenpaineen kohoamiseen. *HFE*-geeni tuottaa solukalvon proteiinia, joka vaikuttaa raudan siirtymiseen soluun. Mutaatio *HFE*-geenissä heikentää proteiinin toimintaa ja tämä voi johtaa raudan kertymiseen elimistöön. Uusimpien tutkimusten mukaan rauta-aineenvaihdunnan häiriö voi muiden vakavien komplikaatioiden lisäksi johtaa verenpaineen kohoamiseen. Verenpainetauti on hyvin yleinen sairaus ja verenpainelääkitystä käytetään laajasti, mutta hoidolliset tavoitteet saavutetaan huonosti. Tämän tutkimuksen tavoitteena oli selvittää geenien *STK39*, *SLC7A1* ja *HFE* yhteyttä verenpainetautiin suomalaisessa kohortissa ja tutkia säännöllisten terveystarkastusten vaikutusta painoindeksiin ja verenpaineeseen 15 vuoden seuranta-ajan kuluessa.

Tutkimuksen mukaan *STK39*-geenin rs6749447 polymorfia ja *HFE*-geenin rs1799945 polymorfia olivat yhteydessä suurentuneeseen verenpainetautiriskiin. *SLC7A1*-geenin rs41318021 polymorfialla ei ollut yhteyttä verenpainetautiin. Säännöllisten terveystarkastusten teho lähtötilanteessa lievästi ylipainoisten keskuudessa oli odotettua heikompi.

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# LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by Roman numerals I-IV:

I. Määttä KM, Nikkari ST, Lähteelä KH, Palmroos PB, Kunnas TA (2013). A functional variant in the serine-threonine kinase coding gene is associated with hypertension: a case-control study in a Finnish population, the Tampere adult population cardiovascular risk study. *Journal of Hypertension* 31:516-520

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# ABBREVIATIONS

BMI	body mass index
BP	blood pressure
CAT	Cationic amino acid transporter
CI	confidence interval
CHD	coronary heart disease
CVD	cardiovascular disease
DBP	diastolic blood pressure
EO	endogenous ouabain
ER	endoplasmic reticulum
GWAS	genome-wide association study
HAMP	hepcidin
HFE	a non-classical MHC class I molecule
HDL	high-density lipoprotein
HJV	hemojuvelin
LDL	low-density lipoprotein
NO	nitric oxide
OR	odds ratio
PCR	polymerase chain reaction
PHE	periodic health examination
RAA	renin-angiotensin-aldosterone system
ROMK	renal outer medullary K channel
RCT	randomized controlled trial
SBP	systolic blood pressure
SLC	solute carrier family 7 member 1

SNP	single nucleotide polymorphism
STK39	serine-threonine kinase 39
TAMRISK	Tampere adult population cardiovascular risk study
TfR2	transferrin receptor 2
WNK	with no lysine kinase

# 1 INTRODUCTION

High blood pressure is a significant health issue in all world regions. In year 2000 the prevalence of hypertension was approximately 26.4 % worldwide. It has been estimated that percentage will be 29 % in 2055. Additionally due to rise of life expectancy the amount of people having hypertension is estimated to increase by 24 % from 333 to 413 million in economically developed countries by the year 2025. (Chockalingam, Campbell, & Fodor, 2006; Kearney et al., 2005) Elevated blood pressure is a leading risk factor for mortality and morbidity globally and it caused 10.4 million deaths in 2010 (Lim et al., 2013).

Cardiovascular disease (CVD) is the major cause for premature deaths in Europe and the most significant risk factor for CVD is high blood pressure (Lim et al., 2013; Perk et al., 2012). Hypertension raises the risk of cardiovascular complications to two- or three-fold (Padwal, Straus, & McAlister, 2001). Lifestyle factors play a key role in reducing blood pressure, 75 % of CVD mortality could be prevented by lifestyle changes. According to this in many European countries the CVD mortality has fallen considerably and now worldwide over 80 % of CVD mortality occurs in developing countries (Perk et al., 2012). Between years 1980 and 2008 systolic blood pressure (SBP) decreased by 0.8 mm Hg in men and 1.0 mm Hg in women globally per decade. SBP is highest in low- and middle-income countries (Danaei et al., 2011). The major contributors to hypertension in Western countries are overweight, physical inactivity, high salt intake and low potassium intake. (Geleijnse, Kok, & Grobbee, 2004) According to twin studies the genetic component of blood pressure is approximated as 30-50 % (Fagard et al., 1995; Kupper et al., 2005; Luft, 2001). Genes behind hypertension have been intensively studied and blood pressure has mainly a complex non-Mendelian mode of inheritance. There are also several rare mutations which cause syndromes of hypo- or hypertension and which are inherited in a clear Mendelian way. (Padmanabhan, Caulfield, & Dominiczak, 2015)

Hundreds of genetic loci affecting blood pressure have been found and until year 2017 they explain 11 % of heritability of blood pressure (Evangelou et al., 2017). Genetic effects may probably constitute hundreds of genes and additionally environmental and behavioural factors have also a crucial role. (Franceschini & Le, 2014; O'Shaughnessy, 2009)

## 2 AIMS OF THE STUDY

Blood pressure is known to have genetic background, but only part of it has been revealed this far. Large studies have been conducted worldwide. Part of the results are controversial and therefore more data is required. New technologies have been established and progress is nowadays fast. Still more studies are needed. Blood pressure is a major health issue globally causing cardiovascular consequences. While great amount of hypertensives are without medication and are not aware of their blood pressure, family component would help the prevention work. Our aim was to study the association of three genetic polymorphisms, in *STK39*, *SLC7A1* and *HFE* with hypertension among 50-year old Finnish subjects. This data was collected from periodic health examinations (PHE). We utilized same collected data for observing the effectiveness of PHE in primary prevention of hypertension among obese.

The aims were:

1. to investigate the association of rs6749447 in *STK39* gene on hypertension
2. to study the association of rs41318021 in *SLC7A1* gene on hypertension
3. to clarify the effect of rs1799945 in *HFE* gene polymorphism on hypertension in Finnish population
4. to evaluate the effectiveness of periodic health examinations on hypertension among obese Finnish people.

## 3 REVIEW OF LITERATURE

### 3.1 Hypertension

Hypertension has been defined as a mean blood pressure 140/90 mm Hg or above (Lewington et al., 2002). This value is for clinic blood pressure, while blood pressure measured at home should be lower, 135/85 mm Hg or below. 2 measurement readings are required with interval of at least 1 minute. Also ambulatory blood pressure can be measured to define blood pressure level. Blood pressure is measured automatically usually for 24 hours, in every 15 or 30 minutes. Averages of daytime and night time are calculated. (Pickering et al., 2005) New definition for hypertension includes two categories, stage 1 and stage 2. In stage 1 blood pressure is 130-139/80-89 mm Hg and in stage 2 blood pressure is 140/90 mm Hg or above. (Whelton et al., 2018)

There is a direct relationship between blood pressure and the risk for developing coronary artery disease and stroke. Other risk factors for coronary heart disease (CHD) include age, male gender, having first degree relatives with CHD, unhealthy diet, physical inactivity, smoking, diabetes, elevated cholesterol levels and psychosocial stress. Approximately 87-100 % of people having coronary heart disease have been exposed to at least one of these risk factors. (Greenland et al., 2003; Perk et al., 2012; Whelton et al., 2018) Lower than 140/90 mm Hg blood pressure level treating targets have been suggested. A meta-analysis including one million adults and 61 prospective observational studies of blood pressure suggested 115/75 mm Hg as a threshold value when considering the increased risk for cardiovascular consequences. (Lewington et al., 2002). In a SPRINT-study blood pressure levels below 140 mm Hg and 120 mm Hg were compared. Lower blood pressure goal resulted in significantly lower cardiovascular events, both fatal and not fatal. Heart failure risk was 38 % lower during 3 years follow-up period with intensive treatment among subjects at high risk for cardiovascular events. Number needed to treat to prevent one primary outcome event was 61. (SPRINT Research Group, 2015) In the ACCORD-study which was conducted among patients having type 2 diabetes mellitus lowering systolic blood pressure below 120 mm Hg instead of below 140 mm Hg did not result in less major cardiovascular events (ACCORD Study Group,

2010). In both of these studies more intensive blood pressure treatment resulted in higher rates of adverse events such as hypotension, kidney failure and electrolyte abnormalities. (ACCORD Study Group, 2010; SPRINT Research Group, 2015)

Many subjects having elevated blood pressure are obese, have diabetes or dyslipidemia and even though the occurrence of hypertension in isolation is below 20 %, the risk factors may have interactions. Therefore overall risk of hypertensive patients for cardiovascular consequences may rise even though the blood pressure is only moderately elevated (Kannel, 1996; Perk et al., 2012). According to a large meta-analysis of Berry et al. (2012) the risk factor profile was considered optimal with untreated blood pressure below 120/80 mm Hg, total cholesterol level less than 4.7 mmol per litre and subject being a non-smoker and not having diabetes. The data for meta-analysis involved a total of 257,387 subjects. (Berry et al., 2012) The risk of cardiovascular disease of subjects having high-normal blood pressure defined as 130-139/85-89 mm Hg has been reported to be 2.5 for women and 1.6 for men when compared with normal blood pressure (Vasan et al., 2001).

Age affects the blood pressure markedly. From age 30 to 65 the blood pressure rises on average 20/10 mm Hg (Kannel, 1996). This elevation raises the risk for cardiovascular consequences, because of the age of 40-69 years a rise of 20/10 mm Hg in blood pressure has been associated with twofold risk for stroke death rate and other vascular causes. In middle and old age blood pressure is strongly and directly associated with vascular mortality. (Lewington et al., 2002) After the age of 60 increasing pulse pressure and decreasing diastolic blood pressure are surrogate measurements for the stiffness of large arteries. If hypertension is left untreated, it may lead to a vicious cycle of accelerated hypertension, because the large artery stiffness further increases due to high blood pressure. (Franklin et al., 1997) Rise in blood pressure due to age may be avoided in an isolated community such as among nuns (Timio et al., 1988) or forager-horticulturalists (Gurven et al., 2012). Suggested factors causing the difference include dietary factors, adiposity, activity and psychosocial stress, so called “modernization” factors (Gurven et al., 2012). Therefore healthy lifestyle has a crucial role in preventing elevated blood pressure.

Recommendations for primary prevention of hypertension include moderate physical activity, maintaining normal body weight, limited alcohol consumption, reduced sodium intake, adequate potassium intake and diet reduced with saturated and total fat (Whelton et al., 2002). Only 38.8 % of hypertensive patients had their blood pressure SBP < 140 mm Hg and diastolic blood pressure (DBP) < 90 mm Hg in a study by Banegas et al. (2011). 94.2 % of hypertensives had medication for hypertension. So below 40 % of hypertensive patients attained treatment goals. This

study included 5559 hypertensive subjects from different European countries, Finland was not included. (Banegas et al., 2011)

World Health Organization (WHO) has set targets for years 2010-2025 and the aim is 25 % relative reduction in the prevalence of raised blood pressure. Other aims include 10 % relative reduction in alcohol consumption, 30 % relative reduction in salt intake, 10 % relative reduction in prevalence of insufficient physical activation and halt the rise of obesity which all have an effect on blood pressure decrease. (World Health Organization, 2013)

The prevalence of hypertension in developed countries according to a meta-analysis including 44 research studies was 40.8 % for men and 33.0 % for women. (Pereira et al., 2009) According to the data from 3128 participants of the Framingham Heart Study increased blood pressure in adulthood was associated with 5 years lower total life expectancy when compared with normotensive people. Normotensive subjects also survived 7.2 years longer without cardiovascular disease compared with hypertensives. (Franco et al., 2005)

According to a large Finnish study blood pressure (both SBP and DBP) fell significantly during the years 1982-2007 in Finland. The study included 16174 participants at the age of 25-64 years. From 1982 to 2007 the percentage of hypertensive men fell from 63.3 % to 52.1% and for women from 48.1 % to 33.6 %. The trend remained for women also during the last 5 years of survey, but for men no further decline was observed. Good progress in prevention and treatment of hypertension has been made in Finland, but improvements are still needed. (Kastarinen et al., 2009) In 2011 46 % of women over 30 years and 53 % of men over 30 years were hypertensive in Finland, which is equivalent to 38 % of all women and 39 % of all men. To reach WHO aims these values should decrease from 38 % to 28 % for women and from 39 % to 29 % for men until year 2025. (Laatikainen, Jula, & Jousilahti, 2015)

Hypertension is common, but the awareness among patients is poor in many cases. Patients have hypertension without knowing their condition. According to reported studies the awareness of hypertension has been 49.2 % and 61.7 % for men and women respectively. Treatment and actual control of hypertension was 29.1 % and 10.8 % respectively for men and 40.6 % and 17.3 % for women. (Pereira et al., 2009) In a Finnish study in 2007 68 % of all hypertensives knew their disease. From those who were aware of their disease, only 52 % was treated with antihypertensive drugs and only 37 % of those had normal blood pressure.(Kastarinen et al., 2009)

Mechanisms of hypertension have been widely studied and they are here reviewed only very briefly. Essential mechanisms are reviewed by Coffman (Coffman, 2011).

Blood pressure is defined according to Ohm's law and fluid dynamics as pressure = flow x resistance, in which flow depends on cardiac output and also blood volume. Resistance depends on the contraction of arteries and arterioles. Cardiac output depends on end-diastolic volume of the heart, myocardial contractility and heart rate. (Guyenet, 2006) Both heart rate and contraction and arterial contraction are greatly modified by central and sympathetic nervous systems (Coffman, 2011). Also kidneys and hormonal regulators play an important role in the regulation of fluid and electrolyte balance and keeping the homeostasis of blood pressure. (Coffman, 2011; Cowley, 2006)

There have been suggestions that hypertension could have also an inflammatory background. Higher C-reactive protein levels have been associated with elevated blood pressure (Cheung et al., 2011; J. H. Lee et al., 2010; Sesso et al., 2003) This reflects a state of low grade chronic inflammation. C-reactive protein levels result from activation of cells of the immune system and vascular endothelium (Sesso et al., 2003). Therefore it is assumed that inflammation could have a role in development of hypertension (J. H. Lee et al., 2010).

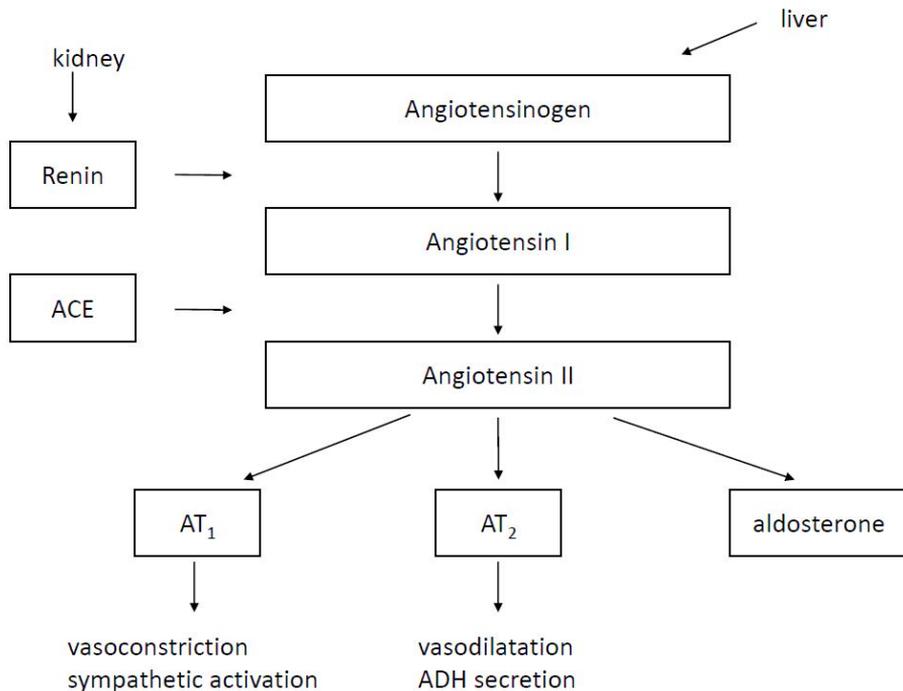
In summary recommendation of blood pressure is below 140/90 mm Hg, but lower pressure levels have been widely studied. There is evidence that preventing cardiovascular consequences lower blood pressure might be more favourable. There are also disadvantages while lower blood pressure levels require more medication and this may rise hypotension and kidney failure risk. Main factors for primary prevention of hypertension includes avoiding overweight, low salt intake, physical activity, low alcohol consumption, low sodium intake and adequate potassium intake. Hypertensive patients reach their blood pressure goals very poorly. Approximately 40 % of Finnish people are hypertensive and therefore the disease burden is remarkable. Hypertension lowers life expectancy and raises the risk for cardiovascular disease. Awareness is also poor, so progress in prevention and treatment is needed.

## 3.2 Environmental factors in hypertension

### 3.2.1 Overweight

Hypertension is strongly associated with obesity (Kotsis et al., 2005; Molenaar et al., 2008; Stabouli et al., 2005). According to Mathieu et al. (2009) 65-78% hypertension

cases are attributed to obesity (Mathieu et al., 2009). The mechanisms include activation of sympathetic nervous system and renin-angiotensin-aldosterone system (RAA). See Figure 1. These lead to abnormal sodium retention and cause elevation of arterial pressure (Rahmouni et al., 2005). Other suggested mechanisms are increased renal tubular sodium reabsorption and impaired pressure natriuresis (Hall, Brands, & Henegar, 1999).



**Figure 1.** Renin-Angiotensin system cascade. Renin is originated from kidney. Angiotensinogen is converted first to Angiotensin I and further Angiotensin II. Angiotensin receptor type 1 (AT1) induces vasoconstriction, endothelial dysfunction and inflammation. AT2 receptor counteracts these changes. Figure is modified from Te Riet et al. 2015 and Sahni et al. 2015. (Sahni, Asrani, & Jain, 2015; Te Riet et al., 2015)

Mechanism leading to sympathetic nervous system activation in obesity is assumed to involve elevated circulating leptin level and activation of the melanocortin system in the central nervous system. Other contributing factors include reduced NO formation, baroreflex dysfunction, increased angiotensin II levels and reduced

adiponectin and ghrelin levels. (da Silva et al., 2009) Long-term sympathoactivation may raise blood pressure by causing peripheral vasoconstriction and also by increasing renal tubular sodium reabsorption. Also hyperinsulinemia may have a role in over activity of the sympathetic nervous system. (Rahmouni et al., 2005) Obesity impairs the function of kidneys. Fat tissue around kidneys cause compression effect activating nervous systems and renin-angiotensin-aldosterone system. Renal tubular sodium reabsorption increases and it impairs pressure natriuresis in the kidneys. This leads to hypertension. (Hall et al., 2015) According to clinical studies BMI increase for one unit increases diastolic blood pressure 0.6 mm Hg for women and 1.0 for men. (K. Liu et al., 1996) Obesity increases also markedly the risk for the incidence of cardiovascular disease. The risk factor for cardiovascular disease is 1.2-2.1 for obese (Padwal et al., 2001).

In German population the prevalence of hypertension was 34.3 % among average weight subjects and 60.6 % among overweighted (Bramlage et al., 2004). Obesity has risen markedly during the 20 years period from 1980 to 2000 also among Finnish people. Trend towards more severe grades of obesity has also occurred. Prevalence of obesity (BMI >30) has risen from 11.3 % to 20.7 % for men and from 17.9 % to 24.1 % for women. Among low educated 25 % of men and 28 % of women were obese. The educational gradient among men has diminished slightly, because the most prominent increase in BMI has occurred among well-educated men. (Lahti-Koski et al., 2010) Mean BMI in Finland was 27.2 kg/m<sup>2</sup> for men and 26.5 kg/m<sup>2</sup> for women in 2007 (Vartiainen et al., 2010). Until the year 2012 the 40 years of rise in BMI seemed to be levelling off, while BMI for women remained unchanged and for men showed only mild increase during 2007-2012. (Borodulin et al., 2015)

Direct association exists between BMI and arterial blood pressure. Anyhow not all obese subjects are hypertensive. Therefore there may be genetically determined variation in the response of blood pressure to weight gain and also initial blood pressure before the weight gain. (Hall et al., 1999) It is assumed that there are protective factors against hypertension. These factors are not known, but it is assumed that gene-environment interactions and epigenetic mechanisms may play a role. Also differences in nutrition, gut microbiota, amount of exercise and even exposure to sun light are proposed to have a role in prevention of hypertension. (Kotsis et al., 2015)

In conclusion overweight is closely connected with hypertension and obesity is a rising problem. There are many possible mechanisms behind overweight causing hypertension. Because 65-78 % of hypertensives are overweight, avoiding weight gain is important when preventing hypertension.

### 3.2.2 Sodium and potassium

High salt intake is one of the key factors producing high blood pressure, see reviews by (Karppanen & Mervaala, 2006; Meneton et al., 2005; Ritz, 2010). Blaustein et al. 2012 reviewed the assumed mechanism behind high salt intake and hypertension. Endogenous ouabain (EO), which is the Na<sup>+</sup> pump ligand, plays a significant role. EO is secreted by brain and adrenals and it promotes blood pressure rise both centrally and peripherally. Elevated Na<sup>+</sup> concentration in cerebrospinal fluid leads increased sympathetic nerve activation and vasoconstriction. Other factors including this assumed complicated network are hypothalamic signalling chain consisting of aldosterone and angiotensin II among others and Ca<sup>2+</sup> -signalling pathway. (Blaustein et al., 2012)

In the large INTERSALT study, comprising of 32 countries in America, Europe, Asia, Africa and the Pacific, percentage of hypertensives varied markedly. Lowest amount was below 1.0 % in the countries where salt intake was low, compared to countries with high salt intake where amount of hypertensives was 17.4 % (Stamler et al., 1991). In four remote populations in the INTERSALT study the average blood pressure was low and hypertension was nearly absent. Also no rise of blood pressure due to age was observed. (Carvalho et al., 1989; Hollenberg et al., 1997) Meta-analysis of randomized controlled trials (RCTs) of the effects of non-pharmacological interventions showed that for hypertensives the effect on systolic blood pressure was with salt restriction -2.9 mm Hg, by weight loss -5.2 mm Hg and stress control -1.0 mm Hg (Ebrahim & Smith, 1998).

Genetic effects on blood pressure include salt sensitivity as reviewed by (Sanada, Jones, & Jose, 2011). Some individuals react on NaCl by increasing blood pressure more than others. Therefore salt restriction is more beneficial for salt sensitive individuals. Anyhow increased salt intake is an independent risk factor for cardiovascular diseases. (Sanada et al., 2011)

High dietary K<sup>+</sup> reduces blood pressure. The mechanism is not well known, but it is assumed that K<sup>+</sup> impacts due to a physiological switch, which alters kidneys to either conserve Na<sup>+</sup> or excrete K<sup>+</sup>. (Welling et al., 2010) The intake of sodium among Americans is double the amount recommended and at the same time intake of potassium is only half of the recommended. This leads to potassium-to-sodium ratio below 1:2, while the guidelines recommend a ratio of over 5:1. (Houston, 2011) It has been approximated that increasing the potassium intake to 4.7 g/d would lower systolic blood pressure with 1.7-3.2 mm Hg in Western countries. The same effect

could be achieved with sodium intake reduction from 9 to 5 g/d. (van Mierlo et al., 2010)

In conclusion sodium intake is too high and potassium intake is too low in industrial countries. When no sodium is used the age-dependent blood pressure rise does not occur. Anyhow the potassium-to-sodium ratio is important.

### 3.2.3 Exercise

Regular physical activity reduces the risk of hypertension. This result has been observed regardless of the level of obesity and for both men and women. (Hu et al., 2004) Aerobic exercise done according to recommendation lowers systolic blood pressure approximately 5-7 mm Hg in hypertensive adults. The recommendation includes moderate intensity aerobic exercise 30 minutes per day preferably every day and dynamic resistance exercise 2-3 times per week. (Pescatello et al., 2015)

Long-term aerobic exercise has been studied to be effective in reducing blood pressure comparable with drugs. Decrease in systolic blood pressure was 9.2 % after three years of exercise, which is comparable to the effect of drug therapies (3.2 % - 16.6 %) during same time period. Possible mechanisms underlying the blood pressure lowering effect include peripheral vasodilatation and increased tissue perfusion (Ketelhut, Franz, & Scholze, 2004). Also Martin et al. (1990) found evidence that aerobic exercise has an independent blood pressure lowering effect (Martin, Dubbert, & Cushman, 1990). A meta-analysis of 13 prospective cohort studies reported the effect of physical activity on hypertension risk. It was found that there was an inverse dose-response association between blood pressure and levels of physical activity. Recreational physical activity was associated with decreased risk of hypertension (RR:0.81-0.89; 95 % confidence interval 0.76-0.94) depending on the level of activation (high/moderate) Association was not significant with occupational physical activation.(Huai et al., 2013)

### 3.2.4 Smoking and alcohol consumption

Although smoking causes instant blood pressure rise, the results of long time average blood pressure levels compared with non-smokers have been inconsistent. Evidence about association is not strong in large studies and several confounding factors exist in different studies, such as alcohol consumption and socioeconomic factors (Primates et al., 2001). Smoking is a significant risk factor for coronary heart disease

in hypertension (Fagard et al., 1995). Jatoi et al. (2007) showed that cigarette smoking was associated with aortic stiffness and the wave reflection in aorta. These effects are reversible, but it may take over a decade to achieve levels of nonsmokers (Jatoi et al., 2007). The risk factors for cardiovascular disease are reported to be elevated among smokers, 1.4 for smoking men and 2.2 for smoking women. For smokers the risk factor was proportional to the number of cigarettes smoked and the deepness of inhalation. (Padwal et al., 2001) Nakamura et al. (2008) found that raised blood pressure and smoking have synergistic effects, while rise of 10 mm Hg of SBP and smoking increased the risk for CVD for 15 % more when compared with non-smokers. (Nakamura et al., 2008)

According to Benowitz and Sharp (1989) smokers had lower blood pressure than non-smokers and magnitude of blood pressure was inversely related to the cotinine concentration of serum. Cotinine is a metabolite of nicotine and it seems to have depressor effects on blood pressure. Cotinine remains in serum longer period than nicotine. (Benowitz & Sharp, 1989) On the other hand in the study of Bowman et al. (2007) cigarette smoking had modest connection with increased risk of hypertension in women who smoked at least 15 cigarettes per day. (Bowman et al., 2007) According to Niskanen et al. (2004) stopping smoking decreases cardiovascular risk, but causes weight gain, which can offset the benefit gained and no change in risk on hypertension may be seen (Niskanen et al., 2004). In the study of Lee et al. (2001) cessation of smoking resulted in increased blood pressure even when weight gain had been taken into account (D. Lee et al., 2001). In the study of Liu et al (1996) cigarette smoking was negatively associated with blood pressure (K. Liu et al., 1996).

According to a large meta-analysis of randomized controlled trials reduction of alcohol consumption of heavy drinkers (mean baseline alcohol consumption 3-6 drinks per day) lowered blood pressure significantly. Mean values for reduction were -3.31 mm Hg and -2.04 mm Hg for systolic and diastolic blood pressure, respectively. (Xin et al., 2001) Fuchs et al. (2001) have reported that heavy use of alcohol is a risk factor for hypertension, but with lower alcohol consumption levels the results are inconsistent and dependent of race, gender and age (Fuchs et al., 2001). Social factors such as education and income are considered as confounding factors when studying the correlation between alcohol consumption and blood pressure (Halanych et al., 2010). The relationship between alcohol consumption and blood pressure rise has been known from year 1915 and it has been estimated that 10 % of the burden of hypertension in the United States is caused by alcohol consumption (Whelton et al., 2018).

In conclusion smoking leads instant blood pressure rise, but long term effects are not that clear, because there are many covariates. On the other hand stopping smoking may result in weight gain and blood pressure rise. Heavy alcohol use raises blood pressure while the results for modest use are more inconsistent. Alcohol and smoking raise the risk for cardiovascular consequences and therefore are part of prevention of disease burden. High alcohol consumption and smoking are related with other unhealthy life habits like physical inactivity and unhealthy diet. There are also other dietary factors affecting hypertension. Glycyrrhetic acid in liquorice causes mineralocorticoid receptor activation and may lead to hypertension. The effect varies individually. (Van Uum, 2005) In contrast calcium has a lowering effect on blood pressure (van Mierlo et al., 2006).

### 3.3 Genetic factors in hypertension

#### 3.3.1 Overview

Genetic background of hypertension has been widely studied and hypertension seems to be a complex, multi-factorial trait. Identification of susceptibility genes could promote recognizing subjects at high risk for developing hypertension at the early stage. Individuals having family history of on early age appearing hypertension have 2.5-fold risk for hypertension when comparing with controls (Oikonen et al., 2011).

Monogenic syndromes causing hypertension have been the starting point of research on hypertension genetics. So far 8 different Mendelian syndromes, which cause hypertension are found. There are 12 genes behind these syndromes and all the genes affect at least one of two metabolic pathways: renal sodium metabolism and steroid hormone metabolism. (Ehret et al., 2011; Ehret & Caulfield, 2013) Monogenic hypertension regardless of the mechanism leads to increase in sodium reabsorption, increased volume and decrease in renin activity in plasma. Increased renal sodium reabsorption causes Liddle's syndrome or Gordon's syndrome. Deficiency of enzymes regulating steroid hormone synthesis or catabolism causes congenital adrenal hyperplasia or apparent mineralocorticoid excess and increased aldosterone synthesis. Glucocorticoid remediable aldosteronism causes suppressed renin release. (Simonetti, Mohaupt, & Bianchetti, 2012) Maas et al. (2015) have reported additional mendelian hypertension type, missense mutation in *PDE3A*,

which encodes cyclic GMP and AMP phosphodiesterase. Mutation leads to arterial remodelling, hyperplasia of vascular smooth muscle cells, and increase in peripheral vascular resistance. (Maass et al., 2015)

In twin research heritability of resting blood pressure is estimated to be 48-60 % for systolic blood pressure and 34-67 % for diastolic blood pressure in a Dutch cohort (Hottenga et al., 2005). Same values for Australian twin cohort were SBP 19-56 % and DBP 37-52 % respectively (Hottenga et al., 2006). There is also evidence that some genes affecting hypertension are age-dependent (Jin et al., 2011). Jin et al. (2011) observed that mitochondrial fusion-regulating gene OPA1 is associated with hypertension in an age-dependent manner. This is due to altered mitochondrial dynamics when aging. Continuous oxidative stress and changes in biomolecules cause altered gene expression, genomic instability and mutations. Increased oxidative stress leads to endothelial dysfunction and also vascular inflammation is observed in aging. (Rammos et al., 2014; Ungvari et al., 2010) These changes may lead to so called age-specific hypertension gene-effects when gene expression profile alters. (Rammos et al., 2014) Until year 2016 below 4 % of the genetic background of hypertension was explained (Ehret et al., 2016). Percentage has been rising rapidly, while in 2017 it was already 11 % (Evangelou et al., 2017).

When hypertension development occurs after the age of 70 years the family history of hypertension is not remarkable, but in cases below age of 60 years family history becomes more important. Therefore hypertension scans for people over 60 years old reveal less clearly the genetic component of hypertension. (Koivukoski et al., 2004)

The genomic study methods for finding hypertension genes include genome-wide association studies followed by different markers and candidate gene studies. Candidate gene approach bases on finding single nucleotide polymorphisms (SNP). For a review of methods see (Charchar, Zimmerli, & Tomaszewski, 2008). Confidently identifying the gene loci responsible for essential hypertension has been a challenge. Blood pressure associated loci interact with other loci and environment also affects them. (Koivukoski et al., 2004)

### 3.3.2 Genome-wide association studies

Genome Wide Association Studies (GWAS) are widely used for finding out hypertension affecting genes. (Burton et al., 2007; Ehret et al., 2016; Eyheramendy et al., 2009; Franceschini & Le, 2014; Levy et al., 2009; C. Liu et al., 2016; Newton-

Cheh et al., 2009; Y. Wang et al., 2009). GWAS makes it possible to dissect hundreds of thousands SNPs in a one chip, see review by (O'Shaughnessy, 2009). GWAS is an experimental design used to detect associations of genetic variants and traits in population. The method bases on the principle of linkage disequilibrium (LD) on the population level. LD describes the non-random association between alleles at different loci. It can be used to map genetic loci, while environmental exposure inquires other methods. (Visscher et al., 2017) Applications of gene expression and DNA methylation among others can be used (Bell et al., 2011).

GWAS method has been used over ten years. In the beginning there was distrust of the discoveries. The results of GWAS studies in recent years have been robust as reviewed by (Visscher et al., 2017). The poor reproducibility of results has been a problem in GWAS studies, especially in the early years. This is due to differences between populations, matching of cases and controls, stratification in populations and heterogeneity in alleles and genes. (Koivukoski et al., 2004; O'Shaughnessy, 2009; Visscher et al., 2012) There are also remarkable differences in pathophysiological factors between different ethnical groups (Koivukoski et al., 2004). The effect of one gene affecting hypertension is very minor and some studies can also be underpowered for identifying such small effects. Also there can be differences in study design or analytical methods used. (Koivukoski et al., 2004) Delles and Padmanabhan reviewed strengths and weaknesses of GWAS studies (see review by (Delles & Padmanabhan, 2012). The main observations were that GWAS are hypothesis-free and enable discovery of new genes and all meiotic recombinations may be detected at high marker density. The other side is that GWAS require large sample size and are costly. Also phenotypic quality can be poor and for analysing the results robust bioinformatics methods are needed. (Delles & Padmanabhan, 2012) Majority of found variants of GWAS lie within noncoding sequence, approximately 90-95 %. This complicates the functional evaluation and leads to transcriptional regulatory mechanisms involved. It is possible to study transcription factor binding by utilizing tissue-selective enrichment of phenotype-associated variants. (Maurano et al., 2012)

In GWAS studies and especially in meta-analyses the amount of subjects is large, tens or hundreds of thousands (C. Liu et al., 2016; Wain et al., 2011). The studies of Newton-Cheh et al. (2009) and Levy et al. (2009) involved both 2.5 million genetic markers and 30,000 subjects. Study of Wain et al. (2011) had sample size of 74,064 and they found 6 possible gene loci in association with hypertension. The reason for these results lies on the fact that hypertension is modulated by a large number of low-risk variants, which each has only a small effect and low penetrance. (Adeyemo

et al., 2009) Blood pressure is a complex heritable, polygenic phenotype. Common variants have minor allele frequency (MAF) $>0.05$ , low-frequency  $0.01 < \text{MAF} < 0.05$  and rare  $\text{MAF} < 0.01$ . Most of the found variants are common. (Surendran et al., 2016) Monogenic hypertension contributes only to minority of hypertension cases (Rafiq et al. 2010). Also these Mendelian genes have been screened in the general population to reveal possible association with blood pressure. A key finding was a gene *KCNJ1* which codes potassium channel ROMK. (Tobin et al., 2008) All these single genes of hereditary hypertension encode molecules that affect salt balance regulated by kidneys (Welling et al., 2010).

The findings of the GWAS studies vary, in some cases no evidence has been found for hypertension genes. Also variants previously associated could not be repeated (Burton et al., 2007). Levy et al. (2009) found four loci which attained genome-wide significance for systolic blood pressure, six for diastolic blood pressure and one for hypertension (Levy et al., 2009). Org et al. (2009) found an association in a locus rs11646213 near the *SDH13* gene, which encodes T-cadherin adhesion molecule involved in angiogenesis (Eyheramendy et al., 2009).

A large GWAS-study by Ehret et al. (2016) included 128,272 SNPs in 201,529 individuals of European ancestry. As a result 66 blood pressure loci for SBP and DBP were found, 17 of these novel. When these 66 SNPs were enriched in vascular endothelial cells for cis-regulatory elements, the results indicate that effects of blood pressure arise from multiple tissues and organs, not just kidneys. Found BP-associated signals are likely driven by non-coding variants. These signals regulate expression of closely situated genes. The findings include vasodilatation inducers *GUCY1A3*, which encodes guanylate cyclase protein and *ADM* which encodes adrenomedulin. (Ehret et al., 2016) Liu et al. 2016 reported 31 novel loci using meta-analysis of association results and exome-centric single-variants. Also 39 previously reported loci were confirmed. Gene-based associations were reported for three novel genes. Many of the new loci have previously been associated with lipids, metabolic phenotypes and immunologic diseases. Joint analysis included 327,288 individuals. (C. Liu et al., 2016) Study of Surendran et al. (2016) included 350,000 individuals genotyped. Exome chip contained 240,000 rare and low-frequency variants. They performed a single-variant discovery analysis. The association of candidate single nucleotide variants was studied with expression levels of nearby genes and tested in aggregate. Their findings include endothelial protein C coding protein receptor, involved in the blood coagulation pathway and RNA binding motif protein 47 and also collagen alpha chain precursor. Two found variants are involved in blood vessel remodelling. (Surendran et al., 2016) Also adiponectin has been associated with

hypertension and it has potent insulin-sensitizing and antiatherosclerosis actions (Y. Chang et al., 2016). In a recent large GWAS study including over a one million people, 535 novel loci were found. New findings were for example transforming growth factor beta, affecting sodium transport in the kidney. They also found enrichment of blood pressure genes in the adrenal tissue, novel loci for aldosterone secretion and vascular remodelling, tone and signalling. (Evangelou et al., 2017)

Many large GWAS analysis and also meta-analysis have been reported. (Ehret et al., 2016; Franceschini & Le, 2014; Wain et al., 2011) Until the year 2010 8 systolic blood pressure, 11 diastolic blood pressure and 6 hypertension genes had been identified using GWAS studies (Rafiq, Anand, & Roberts, 2010). The amount is remarkably rising. The number of statistically significant BP loci was 71 in October 2016 and 901 in 2017 (Evangelou et al., 2017; C. Liu et al., 2016). GWAS-studies have also been conducted using ambulatory blood pressure (Rimpelä et al., 2018; Tomaszewski et al., 2010).

GWAS studies results provide candidates for further study and also may reveal new mechanisms for hypertension. Inevitably there are a very large number of genes involved. (Johnson et al., 2011) GWAS results can be utilized for example in creating genetic risk score models. In these models genome-wide association data is coupled with functional validation approaches. Associative genes may be enriched in different tissue and cell types such as vascular smooth cells or endothelial cells, where they demonstrated clustering. (Mattson & Liang, 2017) Linkage signal found in GWAS studies may be further analysed using additional microsatellite markers and SNP-markers (Y. Chang et al., 2016).

### 3.3.3 Candidate gene approach

Candidate genes for studies are selected for their presumed role in the pathogenesis of disease. Most obvious candidate genes for hypertension include genes affecting renin-angiotensin-aldosterone system (RAA), salt and water handling, vascular tone regulation, signal transduction and adrenergic pathways. (see review about genetics and hypertension by Delles and Padmanabhan 2012) Advantageous in candidate gene studies is that other studies such as gene expression studies or GWAS can be used for choosing candidate genes and genotyping costs are relatively low. Disadvantages include the small effect of one genetic variant to the blood pressure phenotype which leads to possible lack of power to detect small effects. Also

selection of candidate genes depend on the known pathways. (Cowley, 2006; Delles & Padmanabhan, 2012)

Candidate genes affecting RAA-system have been widely studied and association with hypertension is reported. These include renin, aldosterone synthase, angiotensinogen, angiotensin converting enzyme and angiotensin receptors. (Te Riet et al., 2015; L. Wang et al., 2014) Candidate genes are in many cases found by using GWAS studies and then studied further (Graham et al., 2014). Chang et al. (2007) identified three genes associated with blood pressure using genome-wide linkage and candidate-gene-based association studies. These genes were *ATP1B1*, *RGS5*, and *SELE*. *ATP1B1* encodes subunit of Na,K-ATPase, *RGS5* encodes regulator of G-protein signalling and *SELE* encodes E-selectin, which is an endothelium-specific adhesion molecule. (Y. C. Chang et al., 2007) Also pathway and network methods could be useful for identifying candidate genes and loci associated with hypertension (Adeyemo et al., 2009).

In conclusion genetic component rises the risk for hypertension markedly. Therefore genes behind hypertension are in the interest worldwide. Monogenic syndromes causing hypertension are well recognised and these mechanisms have served as a starting point for further research. Heritability of hypertension is approximated as almost 50 %. Only part of the genetic background has been revealed this far. GWAS studies are widely used. The power of GWAS-studies is high, but the results usually need more research. GWAS can be utilized to find new candidate genes and new mechanisms behind hypertension. There are also other available methods.

### 3.4 *STK39*

*STK39* gene which encodes serine/threonine kinase 39 has been identified as a possible candidate gene associating with primary hypertension (Chen et al., 2012; Fava et al., 2011; Niu et al., 2010; Y. Wang et al., 2009). Association was first observed by Wang et al. (2009). Their study was conducted in an Amish population in Pennsylvania area. Amish population is originated from Switzerland in the early 1700s and they are homogenous lifestyle living population and therefore well suitable for identification of genes of complex diseases. *STK39* gene is located on chromosome 2q24.3. (Y. Wang et al., 2009) Reported SNP's of *STK39* associating with blood pressure are listed in Table 1.

**Table 1.** P-values of different SNP's of *STK39* associated with hypertension.

	population	sample size	alleles (major/minor)	p	Reference
rs6749447	Amish	1745	T/G	0.00001	Wang et al. 2009
	Chinese	1108	T/G		Niu et al. 2011
	British Caucasians	1372	T/G	0.29	Cunnington et al. 2009
rs6433027	Han Chinese	1210	T/C	0.035	Chen et al. 2012
rs3754777	Amish	1850	G/A	0.0001	Wang et al. 2009
	British Caucasians	1372	G/A	0.17	Cunnington et al. 2009
	Belgian	1635	G/A	0.0001	Persu et al. 2016
	Han Chinese	1210	G/A	0.007	Chen et al. 2012
rs35929607	British Caucasians	1372	A/G	0.19	Cunnington et al. 2009
	Swedish	23528	A/G	0.02	Fava et a. 2011

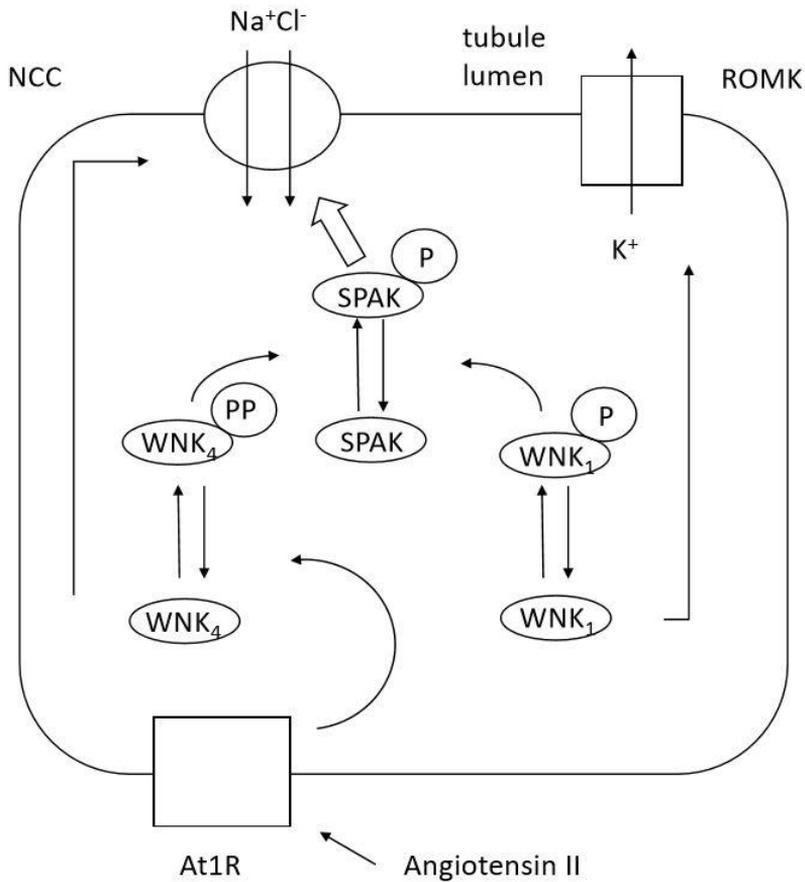
SNP rs3754777 was associated with hypertension in the study of Wang et al. (2009). There has been variation in the results from different populations, while Cunnington et al. (2009) found no association between rs6749447, rs3754777 and rs35929607 with hypertension in a British Caucasian cohort. (Cunnington et al., 2009) In addition some studies have indicated association merely for men or women, but not for the whole study population. In the study of Fava et al. (2011) the association between rs35929607 polymorphism with hypertension was observed only for women in the Swedish population (Fava et al., 2011). This polymorphism caused approximately 21 % increase in hypertension prevalence in women. Chen et al. (2012) found SNPs rs6433027 and rs3754777 in gene *STK39* to be associated with hypertension in males (Chen et al., 2012). The role of *STK39* gene was supported also by the GWAS of Adyemo et al. (2009). They reported 9 SNP's in the gene *STK39* associated with systolic blood pressure and 33 associated with diastolic blood pressure. Two top signals of these were rs2063958 and rs2390639 for SBP and rs11890527 and rs2203703 for DBP. (Adeyemo et al., 2009) According to a meta-analysis including 21 863 hypertensives and 24 480 controls SNP rs3754777 was associated with hypertension among Europeans and East Asians (Xi et al., 2013). Similar result was reported also by Persu et al. (2016) among Belgian cohort (Persu et al., 2016)

*STK39* is part of a multi-gene kinase network (see review by Welling et al. 2010). This network regulates renal Na<sup>+</sup> and K<sup>+</sup> excretion. *STK39* gene (known also as *SPAK* gene) codes Ste20-related proline-alanine-rich kinase. Other factors in the network include with-no-lysine kinases WNK4 and WNK1 and potassium channel

ROMK (renal outer medullary K channel). (Welling et al., 2010) Co-transporters controlling salt reabsorption in the kidneys include  $\text{Na}^+/\text{Cl}^-$  (NCC), and  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  (NKCC1 and NKCC2) co-transporters (Richardson & Alessi, 2008). WNK kinases are upstream activators of *STK39* and *STK39* controls the activity of renal ion co-transporters such as NCC. It is assumed that WNK kinases are the master sensors of  $\text{Cl}^-$  concentration and the activation of  $\text{Na}^+$ -transporters occurs via the kinases SPAK and OSR1. (Richardson & Alessi, 2008)

*STK39* was discovered in 2002 and its role in interaction with cation-chloride cotransporters and WNKs was revealed. During osmotic or oxidative stress cation chloride cotransporters are activated and their activation results in maintaining fluid and ion homeostasis. Cation chloride cotransporters are coupled with  $\text{Na}^+/\text{K}^+$  - ATPase and transport is therefore secondary active. Both *STK39* and OSR1 closely related to it are stress-related Ste20 group kinases and direct interactors of the cation chloride cotransporters. (Piechotta, Lu, & Delpire, 2002) NCC and kidney specific cotransporter, NKCC2 are expressed in epithelial cells of thick ascending limb of Henle's loop and distal convoluted tubule of the nephron where they are the major transport channels for salt reabsorption. (Castañeda-Bueno & Gamba, 2010) *STK39* has also been found acting in brain, where it acts in ion transport regulation and has been associated with autism (Ramos et al., 2008).

The role of *STK39* (*SPAK*) in the kinase network is presented in Figure 2. The kinase network alters the response of kidneys to mineralocorticoid hormone response, which regulates the sodium and potassium conservation and excretion and aldosterone controls both of these processes. (Welling et al., 2010) The reabsorption occurring via NCC and NKCC2 corresponds 20-25 % of the glomerulus filtrate. Most common diuretic drugs regulate the function of NCC and NKCC2. *STK39* modulates the activity of NCC and NKCC2 resulting in arterial hypotension if *STK39* is absent. SNP's may cause increased expression of *STK39* in the kidneys, which increases NCC phosphorylation. As a result blood pressure rises due to promoted salt retention. (Castañeda-Bueno & Gamba, 2010; Glover & O'Shaughnessy, 2011) Therefore according to previous studies *STK39* seems to be a possible target for novel antihypertensive drug therapies (Glover & O'Shaughnessy, 2011; Y. Wang et al., 2009).



**Figure 2.** Angiotensin II activation leads to WNK<sub>4</sub> phosphorylation. WNK<sub>4</sub> activates SPAK, which phosphorylates NCC. This leads to NCC activation and conservation of Na<sup>+</sup>. WNK<sub>4</sub> and WNK<sub>1</sub> are activators of SPAK. WNK<sub>1</sub> acts also with ROMK by inhibiting secretion of K<sup>+</sup>. WNK<sub>4</sub> is activated by AT1R when there is high angiotensinII concentration. WNK, with no lysine kinase; NCC, NaCl cotransporter; ROMK, renal outer medullary K channel. Figure is modified from Welling et al. (2010).

Cunnington et al. (2011) found in their *in vivo* –experiments conducted in peripheral blood cells that polymorphisms in *STK39* gene modified the gene expression. In the group of heterozygous for SNP rs6749447 the G allele was 13 % overexpressed compared to the T allele (Cunnington et al., 2009). Also Wang et al. (2009) observed that the alterations in renal sodium excretion was due to altered gene expression of *STK39*, not the changes in the protein structure.

In conclusion because *STK39* gene has previously been associated with primary hypertension it was validated for our study. Studies had previously been conducted among different populations, but Finland was not included. *STK39* encodes serine/threonine kinase 39, which is a part of network regulating Na<sup>+</sup> and K<sup>+</sup>

excretion in kidneys. *STK39* modulates the activity of NCC and NKCC2, which affects reabsorption in kidneys. Increased expression of *STK39* in kidneys cause promoted salt retention and as a result blood pressure rises.

### 3.5 *SLC7A1*

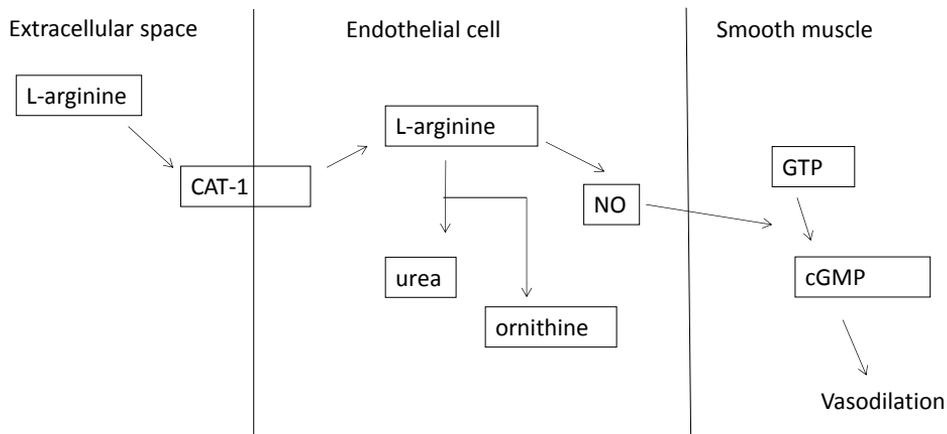
The role of endothelium, the single-cell layer that covers the inner surface of the blood vessels, is significant in regulation of blood pressure as a sensor and also as a modulator (see review by Anderson 2003). Endothelium creates a nonthrombogenic and nonadhesive surface and it also synthesizes vasodilatory substances like nitric oxide (NO) (Ignarro et al., 1987; Yang & Kaye, 2006). Endothelial dysfunction has been demonstrated in hypertension, hypercholesterolemia, atherosclerosis, diabetes, smoking and inflammation (Anderson, 2003; Grover-Piéz & Zavalza-Gómez, 2009). In endothelial dysfunction ability of endothelium for stimulation and therefore to vasodilatation is impaired (Yang & Kaye, 2006).

Yang et al. (2007) studied gene *SLC7A1* (or *CAT-1*), which codes L-arginine transporter, using endothelial-specific transgenic mouse. Study indicated that change in L-arginine transport influences NO production and vascular tone (Yang et al., 2007). This finding provides a link between altered endothelial function, NO metabolism, L-arginine and essential hypertension. Frequency of the functional variant of the *SLC7A1* gene was increased in subjects having essential hypertension. Furthermore altered expression in experimental models resulted in changes in NO production and changes in endothelial function. (Yang et al., 2007)

Cationic amino acid transporters (CATs) mediate the bidirectional transport of the cationic amino acids and support metabolic functions such as NO synthesis. CAT-1 was the first cloned amino acid transporter. (see review by Hatzoglou 2004) *SLC7A1* includes in the human gene family solute carrier 7 which is a cationic amino acid transporter for arginine and lysine uptake in mammalian cells (Hatzoglou et al., 2004). Expression level of CAT-1 varies in different tissues and can be modulated by growth factors, cytokines, nutrients and hormones. (Hatzoglou et al. 2004) One example is insulin, which increases *SLC7A1* gene expression due to increased transcriptional activity in human umbilical vein endothelium (González et al., 2011).

Arginine is a precursor for nitric oxide. Arginine is originated from the diet, endogenous synthesis and also from the protein turnover in the body. For healthy adults arginine is not an essential dietary amino acid due to its sufficient level of endogenous synthesis. Arginine metabolism has been reviewed by Morris et al.

(2007). (Morris Jr, 2007) L-arginine transport system in the cell is presented in Figure 3. Arginine is a substrate of two alternative pathways: for endothelial nitric oxide synthase (eNOS) which acts as a catalyst in nitric oxide (NO) production and for arginase, which converts arginine to ornithine and urea. (Visigalli et al., 2010) Nitric oxide (NO) acts as an antiatherogenic vasodilator. NO is synthesized from arginine or citrulline. Arginine and citrulline increase eNOS protein levels leading to elevation in NO production. (Berthe et al., 2011) Impairment of L-arginine transport has been observed in hypertension. The role of arginine as a key substrate for NO biosynthesis makes it an important factor in the pathogenesis of cardiovascular diseases. (see review by Chin-Dusting 2007) (Chin-Dusting, Willems, & Kaye, 2007)



**Figure 3.** L-arginine transport system in the cell and production of NO. Modified from Chin-Dusting et al. 2007.(Chin-Dusting et al., 2007)

Mechanism by which SNP ss52051869 at the gene *SLC7A1* regulates the gene expression is the alterations in its binding of transcription factor SP1. Major allele contains a consensus sequence for transcription factor SP1 which allows its binding to SP1 while minor allele is not capable to bind. Polyadenylation of *SLC7A1* can occur in two alternative sites. Therefore two mRNA variants with different 3' end untranslated regions (3'UTRs) exist. Minor allele 'T' in rs52051869 is associated with long-form 3'UTR and major allele 'C' tends to accompany only to shorter form of 3'UTR. Minor allele fails to bind SP1 while major allele contains a consensus sequence and is able to bind to transcription factor SP1. SP protein-dependent

transactivation involves binding to GC-rich promoter sequences and also interactions of transcription factor components. Different binding plays important role in gene expression and regulation. (Yang & Kaye, 2009)

The effect of decreased availability of L-arginine on impaired endothelial function is controversy. L-arginine transport has been reported to be impaired in hypertensive and normotensive subjects who have genetic background of hypertension (Schlaich et al. 2004). Based on animal models it is assumed that L-arginine supplementation could enhance the function of endothelium. However, there are no conclusive randomized clinical trials conducted in humans on the subject, possible due to study size. (Bode-Böger, Scalera, & Ignarro, 2007) Schlaich et al. (2004) have reported that for those who had impaired L-arginine transport, the substrate infusion resulted in endothelium-dependent vasodilatation (Schlaich et al., 2004). Extracellular arginine concentration is the major determinant of NO production, not intracellular, in endothelial cells. Intracellular arginine seems to be not capable of utilizing membrane-bound eNOS. Earlier stated “L-arginine paradox” is that intracellular ARG concentration (1 mM) is much higher than Km of NOS (3µM) and therefore increasing the concentration of ARG should have no effect on NO production, but according to earlier studies it has. Error here is that not the intracellular, but the extracellular ARG concentration should be compared, since intracellular ARG is not relevant for NO production in the cell. (Shin, Mohan, & Fung, 2011) Impaired basal production of NO has been observed also in the offspring of hypertensive parents and therefore it seems that endothelial dysfunction does not occur solely as a consequence of hypertension, but instead it may precede the condition. (McAllister & McCance, 1999) Hypertensive patients are reported to have abnormal endothelial function and it is related to NO production either by reduced synthesis, release or diffusion of NO (Panza et al., 1993). Obesity and metabolic syndrome cause dysfunction of L-arginine influx into platelets and is correlated with insulin resistance. This may be due to depletion of intraplatelet amino acid concentration or alterations in platelet membrane properties. (Assumpção et al., 2010)

In conclusion Nitric oxide (NO) is an important vasodilator in the body. NO is produced from L-arginine. *SLC7A1* encodes L-arginine transporter and therefore affects the concentration of L-arginine in the body. Changes in *SLC7A1* may cause impaired transport and lead to lower levels of NO. This causes vasoconstriction and may rise blood pressure. *SLC7A1* is one of the target genes behind hypertension.

### 3.6 HFE

Iron is essential for body homeostasis because it is needed in many biochemical reactions like DNA synthesis and oxidative phosphorylation and also in oxygen transport system. There is approximately 3-5 g of iron in the human body. Main part of iron is bound to haemoglobin in circulating red blood cells. Iron uptake occurs in duodenum and jejunum. (Canavesi et al., 2012; Means Jr, 2013) Iron is stored in hepatocytes and macrophages within polymers of ferritin (Ganz, 2011).

Approximately 65 % of body iron is bound in haemoglobin, 10 % is in myoglobin in muscles and as enzymes and cytochromes. The rest is stored in the liver, reticuloendothelial system, macrophages and bone marrow. Iron is absorbed at the duodenum. After absorption iron is reduced by ferrireductase enzyme and transported across the intestinal epithelium and internalized in the enterocyte. After exportation across the basolateral membrane iron is bound by plasma transferrin. (Munoz, Garcia-Erce, & Remacha, 2011) Ferroportin is the transmembrane iron exporter, which releases iron into the blood stream from hepatocytes, duodenal enterocytes and macrophages. Ferroportin is mainly expressed on the surface of macrophages and on basolateral membrane of duodenal enterocytes. 20-25 mg iron per day is released by macrophages into plasma, 1-2 mg is released by enterocytes which is originated from dietary iron. Ferroportin expression is controlled in response to iron deficiency, hypoxia, heme iron and inflammatory factors. (Collins, Wessling-Resnick, & Knutson, 2008; Enculescu et al., 2017)

Iron excretion is very limited and therefore excess iron may accumulate in the body promoting noxious free radical reactions (Pietrangelo, 2007). Excess iron in the blood saturates the buffering capacity of serum transferrin, which leads to appearance of reactive forms of iron in the blood. This pro-oxidant iron causes oxidative organ damage in the liver and other organs causing cirrhosis, hypogonadism, diabetes, cardiomyopathy, artropathy and skin pigmentation and raises the risk for hepatocellular carcinoma. (Crowover & Covey, 2013; Crowover & Covey, 2014; Ganz, 2011; Murphy & Oudit, 2010; Pietrangelo, 2015)

Iron regulation is complex as reviewed by Muckenthaler et al. (2017). Ferroportin is the exporter at the plasma membrane. Heparin is found to be a key regulator of systemic iron homeostasis. Heparin acts in posttranslational ferroportin regulation and inhibits iron efflux by binding to ferroportin. This leads to ubiquitination and degradation of ferroportin. HFE is an upstream regulator of heparin. (Muckenthaler et al., 2017; Zacharski, 2010) Heparin has been widely studied recently and also its measurement techniques have been studied to advance diagnosis

and treatment of iron metabolic diseases (Girelli, Nemeth, & Swinkels, 2016). Heparin is synthesized and released from liver according to iron deficiency, hypoxia, heme iron and inflammatory factors (Adams & Barton, 2007; Enculescu et al., 2017; Girelli et al., 2016).

Iron overload is mostly genetic in Western populations due to hereditary hemochromatosis. Hereditary hemochromatosis is caused by polymorphisms in genes *HFE*, hemojuvelin (*HJV*), hepcidin (*HAMP*), ferroportin (*FPN*) and transferrin receptor 2 (*TFR2*). All these polymorphisms cause hepcidin deficiency. Other explanations for iron overload are thalassemias due to secondary iron loading. See reviews by (Crowner & Covey, 2013; Crowner & Covey, 2014; Pietrangelo, 2016) Hemochromatosis is a syndrome caused by toxic effects of excess iron in the body. It is caused by genetic or acquired defect of iron transport into the blood (Pietrangelo, 2015). Classical *HFE*-associated hereditary hemochromatosis is an autosomal recessive disease and most commonly mutated gene polymorphisms are C282Y and H63D. Third, but rare, polymorphism is S65C. H63D has remarkable weaker effect on iron overload than C282Y. Modifying factors added with H63D are assumedly needed for iron overload. (J. N. Feder et al., 1998; Gochee et al., 2002) Majority (80.6 %) of hemochromatosis patients were homozygous for the C282Y polymorphism. Disease penetrance among C282Y homozygotes is reported as 13.5 %. In the general population the carrier frequency for C282Y was 6.2 %, for H63D 14 % and for S65C 0.5 %, consisting both hetero and homozygotes combined. (European Association For The Study Of The Liver, 2010)

An atypical class 1 molecule HLA-H was defined as *HFE* after its discovery in 1996 (J. Feder et al., 1996). Function on *HFE* is not fully understood. It acts in hepcidin regulation with hemojuvelin and transferrin receptor 2 (Muckenthaler et al., 2017). The dominant missense polymorphism of classical *HFE*-associated hereditary hemochromatosis C282Y is caused by a single nucleotide change G to A. This results in change of cysteine to tyrosine in the position of 282 coding disulphide bridge in the MCH class 1 protein. Other polymorphism causes a single nucleotide change C to G, which changes His to Asp at amino acid 63. (J. Feder et al., 1996) Polymorphism C282Y results in configurational changes in protein and incomplete folding. This causes protein retention in the endoplasmic reticulum of a hepatocyte and leads to ER stress. Polymorphisms in H63D protein forms stable complexes with transferrin receptor. This binding lowers receptor binding affinity for transferrin, but the effect on cellular iron content is minor. (Gray, Crowe, & Lawless, 2009) It has been suggested that H63D polymorphism may affect cell function by

altering energy levels, susceptibility to stress and transcription activation in cells (S. Y. Lee et al., 2007).

H63D polymorphism has also been associated with higher risk for type II diabetes mellitus. The assumed mechanism could be the toxic effect of iron on beta cells in pancreas. (Colli, Gross, & Canani, 2011)

There is variation in the frequency of *HFE* gene polymorphisms in different populations. In Indian population C282Y is rare, absent in native Indians, but there are 9-13% of H63D (187C →G) carriers. Dhillon et al. (2012) studied the origin of H63D polymorphism in Indian population and suggested that origin is resulted in population admixture with European chromosomes. (Dhillon et al., 2012) The frequency of H63D polymorphism carriers is 10-20 % in Europe, highest frequencies are near the Mediterranean. Polymorphism is very rare in Africa, and South and Central America (Khusainova et al., 2013; Merryweather-Clarke et al., 1997). In Finland the frequencies for C282Y and H63D polymorphism carriers have been reported as 13.0 % and 20.3 %, respectively (Parkkila et al., 2001).

Due to increase of oxidative stress *HFE* gene polymorphisms also increase the risk of coronary heart disease. (Silva et al., 2010) Serum ferritin levels are affected by gender and environmental factors (Allen et al., 2008; Rivers et al., 2007).

In the study of Ekblom et al (2011) women who were H63D DD homozygotes had higher risk for myocardial infarction. Iron levels for H63D D allele carriers were similar when compared with other groups and therefore mechanism is probably other than iron overload. (Ekblom et al., 2011) Iron overload was not associated with hypertension or left ventricular hypertrophy. (Ellervik et al., 2010) *HFE* H63D polymorphism has been associated with arthropathy, but there was no association with the risk of coronary heart disease (Alizadeh et al., 2007; Pankow et al., 2008; Van Der et al., 2006).

Lead and iron are both absorbed by the same mechanism in the gut. Lead (Pb) has no biological function in the body. The assumed transporters are the divalent metal transporter 1 (DMT1) and ferroportin 1 (FP1). (Zhu et al., 2013) In the study conducted among Chinese lead exposed workers H63D polymorphism carriers had higher blood lead concentration and higher lead amount was associated with a higher body iron content or lower transferrin level. According to this study H63D carriers have excess iron stores and when they are exposed to lead the absorption may promote lead poisoning. (Fan et al., 2014) In the study of Zhang et al. (2010) the relationship between pulse pressure and bone lead amount was linear among H63D polymorphism carriers. This indicates that H63D polymorphism may affect lead toxicity and enhance the environmental factors of cardiovascular diseases. Changes

in pulse pressure were assumed to be independent on iron pathway and occur via oxidative stress or inflammatory processes. (Zhang et al., 2010) In the study of Njajou et al. (2002) there was evidence of modification of the relationship between *HFE* polymorphism and smoking on stroke. This was assumed to be because of increased oxidative stress and damage of vessel wall. The effect was higher than additive. (Njajou et al., 2002)

A large genome-wide association study (GWAS) has given evidence that polymorphism in H63D could associate with blood pressure (Ehret et al., 2011). In some studies H63D polymorphism has been associated with iron overload (Brandhagen et al., 2000) but not with coronary heart disease (Waaen et al., 2002). H63D carriers had higher ferritin levels suggesting modulating effect on iron absorption. H63D polymorphism has been studied to be involved with increased endoplasmic reticulum stress. The assumed mechanism is perturbation of protein folding machinery. (Y. Liu et al., 2011; Santos et al., 2010)

In conclusion iron is essential for body homeostasis and its regulation pathways are very complex. Heparin is an iron regulating hormone, which affects iron levels markedly. *HFE* is known to be an upstream regulator of hepcidin in iron homeostasis. *HFE* polymorphisms C282Y and H63D are known to cause hemochromatosis, which is iron accumulating disease. There is evidence that H63D polymorphism causes cardiovascular diseases. A GWAS study has shown association between hypertension and H63D polymorphism.

### 3.7 Periodic health examinations (PHE)

Already in 1932 modern medicine was seen to shift towards prevention of disease: “an ounce of prevention is worth a pound of cure”. People were educated that they must no longer live to eat, but should eat to live. Also people over 40 years should be looked over if there was something which can be done for his or her health. (Harrison, 1932) In Germany periodic health examinations (PHE) were started in 1925 and they aimed for early diagnosis of diseases (Neustätter, 1934). In Canada PHE were planned by leading insurance companies and Canadian Medical Association. The idea was to detect disease in its incipency so that they could be corrected before it is too late. Even though the costs of PHE were remarkable. (AD, 1930)

Nowadays the main purpose of PHE are still early diagnosis and treatment of manageable diseases. The benefits of PHE seem to be largest for subjects aged 40-54 years. PHE should be focused on major and manageable diseases. (Lin et al., 2011) Periodic health examinations (PHE) generally include subjects' medical history, selected laboratory tests and physical examination. Aims for PHE are also improving patients understanding of health and disease, establish basis for continuous health supervision and provide advice concerning habits affecting health such as smoking. (Breslow, 1959)

The results of the effectivity of PHE are controversial. According to a large meta-analysis, consisting of 16 randomized trials (182 880 participants), the general health checks did not reduce mortality or morbidity. The number of new diagnoses however increased. (Krogsboll et al., 2012) In another study health checks in the beginning and after three years lead to 3.1 % decrease in total cholesterol, 1.9 % decrease in systolic and diastolic blood pressure and 1.4 % decrease in BMI, which all were statistically significant results. Anyhow when diastolic blood pressure was over 100 mm Hg or BMI over 30, no beneficial effect of PHE was shown. Annual rechecks were not more effective than a check every three years. There were shown benefits of PHE that were sustained over three years, but the changes were anyhow minor. These benefits need to be weighed against costs.(Group, Imperial Cancer Research Fund OXCHECK Study, 1995) When nationwide periodic health examinations were studied in Taiwan, it was observed that people who used the preventive services had higher probability to get early treatment for hypertension, diabetes and hyperlipidemia. (Lin et al., 2011) Effects of PHE depend also on attitude of the subject. Not all subjects are interested in participating the voluntary health checks. Also subjects' attitudes and expectations on health examinations vary according to interval and costs of the examinations. (Oboler et al., 2002)

## 4 MATERIALS AND METHODS

### 4.1 Participants

The Tampere adult population cardiovascular risk study (TAMRISK) is a prospective, longitudinal health survey conducted in Tampere, a city with a population of 210 000. In Tampere, the city health care centre has provided regular periodic health examinations (PHE) for all 40 and 50 years old inhabitants since 1980 due to screening and counselling. Participation has been voluntary, but all 40 and 50 years old inhabitants have been invited. During some periods also 35 and 45 years old inhabitants have been invited to participate.

The PHE were held at the Tampere centre's health examination unit and they consisted of one 60 minute session with a public care nurse. Session included questionnaire information and screening tests. Interview was done using a structured questionnaire including questions of health and health-related behaviour. Also current and previous diseases were asked. Counselling was held based on findings of the screening tests and also on topics selected by the subject. Informed consent was obtained similarly with physical examination for use of the data for research. In the physical examination blood pressure was measured using a calibrated mercury sphygmomanometer. Total serum cholesterol, haemoglobin and glucose were analysed in the laboratory from the taken blood samples. Also subjects' height and weight were measured in the physical examination.

The data for TAMRISK study was collected from the periodic health examinations and it includes information of risk factors for hypertension: blood pressure, weight, lipid values, smoking, diabetes, physical exercise habits and family history of cardiovascular diseases. Baseline clinical examinations had taken place in 1988-91, next follow-up in 1993-96 when subjects were 40 years old, third follow-up in 1998-2001 and the last follow-up in 2003-2006 when subjects were 50 years old. DNA for genetic studies was collected from buccal swabs.

Participants received buccal swabs for collecting DNA sample and a permission form to use PHE data by mail separately of the physical examination. The DNA samples were collected during years 2006-2010 and informed consent was obtained

from all participants. The Ethics Committees of the Tampere University Hospital and the City of Tampere approved the study.

Cases of our study were subjects, who had hypertension diagnosed by a physician at the age of 50 years. For each case at least one control was chosen from PHE cohort. Controls were normotensive, same sex and similar smoking habits than cases. Study cohort consisted of subjects having available data of hypertension measurements at the age of 35, 40, 45 and 50. Clinical characteristics of the study population is presented in Table 2.

**Table 2.** Clinical characteristics of study population at the age of 50 years.

Clinical characteristic	Cases (n=447)	Controls (n=771)	p
Age (years)	50 ± 0	50 ± 0	
Body mass index (kg/m <sup>2</sup> )	28.6 ± 5.1	25.4 ± 3.6	< 0.001
Cholesterol (mmol/L)	5.4 ± 1.0	5.4 ± 1.0	0.7
HDL cholesterol (mmol/L)	1.6 ± 0.4	1.7 ± 0.4	0.4
LDL cholesterol (mmol/L)	3.2 ± 0.9	3.2 ± 0.8	0.9
Triglycerides (mmol/L)	1.5 ± 1.2	1.2 ± 0.7	0.03
Glucose (mmol/l)	5.1 ± 1.2	4.8 ± 0.6	0.05
SBP (mm Hg)	143 ± 16	130 ± 15	< 0.001
DBP (mm Hg)	93 ± 9	84 ± 9	< 0.001
Hypertension (%)	100	0	< 0.001
Exercise (at least twice a week) %	66	72	0.06
Diabetes (%)	7	0	< 0.001
Lipid lowering drugs (%)	15	3	< 0.001
Myocardial infarction (%)	2	0	< 0.001
Blood pressure medication (%)	67	0	< 0.001
Gender (male) (%)	60	57	

Data is presented as mean ±SD

## 4.2 DNA genotyping

DNA was extracted from buccal swabs using a commercial kit (Qiagen Inc., USA). Genotyping was performed using allele specific primers (*STK39* and *SLC7A1*) or conventional RFLP (restriction fragment length polymorphism) analyses.

For *STK39* two parallel PCRs were performed for each DNA sample. All tubes contained primer 5'-CCT GTA TTT ACA AGC CCC ACA-3' plus either 5'AGT CTG CTA GTA CTA GAT TAG GAG-3'(for G-allele) or 5'-GAG TCT GCT AGT ACT AGA TTA GGA T- 3'( for T-allele). These primers were otherwise similar, but the last nucleotide in the 3'end was either G or T and another was one nucleotide longer. PCR program: amplification 94°C for 15 min, 34 cycles of 94°C 30 s, 55°C 30s and 72°C 30 s and final extension 72°C 5 min. After amplification, PCR products (193 bp) were run on a 1 % agarose gel electrophoresis and DNA genotypes were called directly according to amplified fragments (G or T or both).

For *SLC7A1* two parallel PCRs were performed for each DNA sample. These contained pri047 (5'-AGT TGT CTG GAG GTG ACC-3), plus either pri049C (5'GCA AGT GAG GCA CAG CCC-3') or 050T (5'GCA AGT GAC GCA CAG CCT-3'), under the same conditions of PCR (94°C for 15 min, 34 cycles of 94°C 30 s, 55°C 30s and 72°C 30 s and final extension 72°C 5 min). After amplification, PCR products were run on a 2 % agarose gel and DNA genotypes were called directly according to amplified fragments (049C or 050T or both). The length of the PCR products was 215bp.

For H63D analysis DNA samples were amplified using primers 5'-ACA TGG TTA AGG CCT GTT GC-3' and 5'-GCC ACA TCT GGC TTG AAA TT-3'. PCR was performed with program: amplification at 94°C for 5 minutes, then 36 cycles of 94°C 30 s, 61°C 30s, and 72°C 30s and then final extension at 72°C for 7 min. Then products (208 bp) were digested with restriction enzyme BcII. Digested products (208 bp = GG, 208 bp, 138 bp and 70 bp = CG and 138 bp, 70 bp = CC genotypes) were separated using 2 % gel electrophoresis.

### 4.3 Statistical analysis

The description data of cases and controls was performed using means and standard deviations for continuous variables and percentages for categorical variables. Comparison between cases and controls was performed using analysis of variance (ANOVA) for continuous variables and Chi-square test for categorical variables. For skewed distribution values the analysis was performed using transformed values to approximately normalize the distribution.

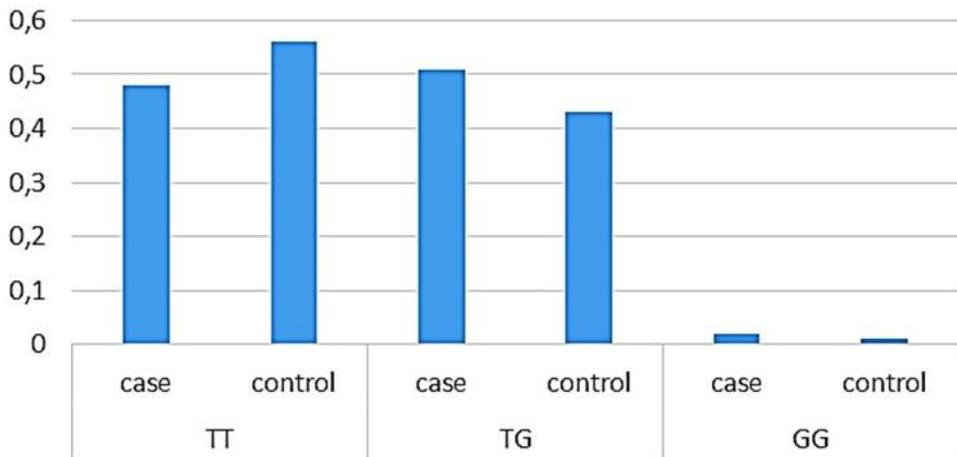
In our study the risk level was set as p value below 0.05 and therefore results in which p value was  $<0.05$  were considered as statistically significant. Confidence interval 95 % was used. Analysis were performed using SPSS 16.0 and 20.0 (SPSS Inc., USA).

To evaluate the effect of gene polymorphisms on the risk of hypertension we used binary logistic regression analysis (Forward Wald). When the differences of blood pressures at the age of 35, 40, 45 and 50 years were analysed we used analysis of variance for repeated measures. The use of hypertension medication was known, but it was not taken into account, while the blood pressure of subjects having medication was not statistically different when comparing to subjects not having medication. In addition information of medication was given by subjects themselves and information was not available for all subjects.

# 5 RESULTS

## 5.1 Association of *STK39* gene with blood pressure

Genotyping of *STK39* gene polymorphism rs6749447 (T>G) was successful for 1158 subjects (404 cases and 754 controls). The frequencies of gene variants were significantly different between cases and controls ( $p=0.026$ ) using Chi-square test. There were significantly more altered gene carriers among cases; 0.51 TG and 0.02 GG compared with 0.43 TG and 0.01 GG among controls (see Figure 4). Due to low number of subjects GG group was included into the TG group for further analysis of the study.



**Figure 4.** Frequencies of *STK39* gene variants in the study population.

The risk for hypertension among G-allele carriers was 1.4-fold compared with controls ( $p=0.006$ , 95 % CI=1.10-1.79). *STK39* gene, BMI class, sex and family history of hypertension were included as explainable variables in logistic regression analysis (stepwise Forward Wald). According to analysis, mutated *STK39* gene carriers had 1.5-fold risk for hypertension. Family history of hypertension increased the risk to 3.5-fold.

BMI associates strongly with hypertension ( $p < 0.001$ ) and therefore the study population was divided according to BMI into subgroups for further analysis.

The association of *STK39* gene variants with hypertension was then calculated separately for average-weight ( $BMI < 25$ ) and overweight ( $25 < BMI < 30$ ) subjects. For average-weight subjects the association of *STK39* gene was even more significant ( $OR = 2.0$ ,  $p = 0.003$ , 95% CI 1.3-3.1), as also for subjects overweight ( $OR=1.6$ ,  $p = 0.001$ , 95% CI 1.2-2.2). In the group BMI over 30 the effect of *STK39* was no longer significant ( $p = 0.832$ , 95% CI 0.6-1.9).

The follow-up data for subjects having hypertension measurements at the age of 35, 40, 45 and 50 available (780 participants) was analyzed using repeated measures –analysis. The average blood pressure of *STK39* polymorphism carriers was higher during the whole 15 years follow-up time,  $P= 0.06$  for systolic BP and  $P=0.016$  for diastolic BP compared to major allele homozygotes.

## 5.2 The effect of *SLC7A1* genetic variant on blood pressure

Genotyping of *SLC7A1* gene polymorphism rs41318021 (C>T) was successful for 1183 subjects. There was no difference between the frequencies of mutated gene carriers of case group and control group ( $p = 0.209$ ). In the hypertension group the frequencies were 0.69 for CC, 0.29 for CT and 0.02 for. The frequencies in the control group were 0.71, 0.26 and 0.03, respectively.

Interpretative factors for hypertension were analysed using logistic regression analysis (stepwise analysis, forward wald). *SLC7A1* genotype, family history of hypertension and BMI, Hb, HDL cholesterol, glucose and triglycerides data at the age of 50 years was included into analysis. As a result BMI, ( $p<0.001$ ,  $OR: 1.19$ , 95 % CI 1.14-1.24, glucose ( $p=0.009$ ,  $OR: 1.37$ , 95 % CI: 1.08-1.75) and family history of hypertension ( $p<0.001$ ,  $OR: 3.28$ , 95 % CI: 2.35-4.56) were associated with hypertension. For analysis BMI was classified as average-weight ( $BMI < 25$ ), overweight ( $BMI < 30$ ) and obese ( $BMI > 30$ ). Glucose was classified as normal or high blood glucose concentration.

Follow-up data for subjects having available measurements at all the ages of 35-, 40-, 45- and 50 years was analysed for cases and controls. There were only 23 subjects with genotype TT and they were combined with CT genotype group for the analysis. Repeated measures analysis showed that subjects having gene variant TT or CT had slightly higher diastolic blood pressure through the follow-up compared with CC allele carriers ( $p=0.047$ ). The difference was most significant at the age of 35 years,

1.4 mmHg. This was also the only age point where there was a statistically significant difference between blood pressure values of cases and controls. This was also for diastolic blood pressure ( $p=0.044$ ) using one way ANOVA analysis. Results remained the same when covariates BMI and glucose were added to the repeated measures model. Subjects having gene variants CT or TT had higher diastolic blood pressure than subjects having genotype CC ( $p=0.021$ ). Difference for systolic blood pressure was not significant ( $p=0.241$ ).

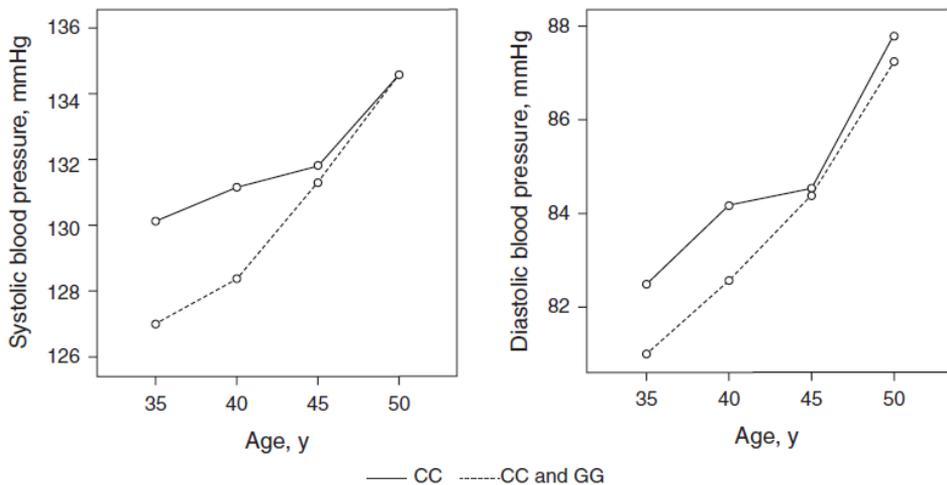
### 5.3 *HFE* gene and blood pressure

Genotyping of *HFE* gene polymorphism H63D was succeeded for 1150 subjects. The frequencies of the different genotypes were 0.74 for CC ( $n= 851$ ), 0.24 for CG ( $n = 280$ ) and 0.017 for GG ( $n=19$ ). Genotype frequencies were significantly different between cases and controls ( $p = 0.04$ ). Frequencies for genotype CC were 0.70 ( $n = 279$ ) for cases and 0.76 ( $n = 572$ ) for controls, CG 0.28 ( $n = 112$ ) for cases and 0.22 ( $n = 168$ ) for controls and GG 0.02 ( $n = 8$ ) for cases and 0.015 ( $n = 11$ ) for controls. As there were only 19 GG variants, the groups GG and GC were combined for further analysis.

Association of H63D polymorphism on hypertension was studied using logistic regression analysis. When association of H63D was included into analysis alone, the risk for hypertension was 1.4-fold for gene polymorphism carriers ( $p= 0.018$ , 95 % CI 1.06 -1.82). When family history of hypertension and BMI were also included into analysis with H63D polymorphism, the adjusted odds ratios (OR) for hypertension were 1.4 for H63D D-allele carriers, 1.2 for BMI and 3.6 for family history of hypertension. For analysis BMI was classified as average-weight ( $BMI < 25$ ), overweight ( $25 < BMI < 30$ ) and severe overweight ( $BMI > 30$ ). In the latter analysis logistic regression analysis (forward conditional) was used. Association was also calculated in subgroups of overweight ( $BMI>25$ ) and obese subjects ( $BMI > 30$ ). For overweight G-allele carriers OR was 1.6 and for obese 4.1.

Blood pressure during the follow-up time was analysed for gene polymorphism carriers and controls. It was clearly shown that both systolic and diastolic blood pressures were significantly different at the age of 35 years. Mean BP among CC carriers was 127/81 mm Hg and among D allele carriers it was 131/83 mm Hg, respectively ( $p < 0.001$  for SBP and  $p = 0.005$  for DBP). The trend of blood pressures during follow-up time is seen in Figure 5. The observed difference in blood

pressures was shown also at the age of 40 years for systolic BP ( $p=0.027$ ), but not at the ages of 45 or 50.

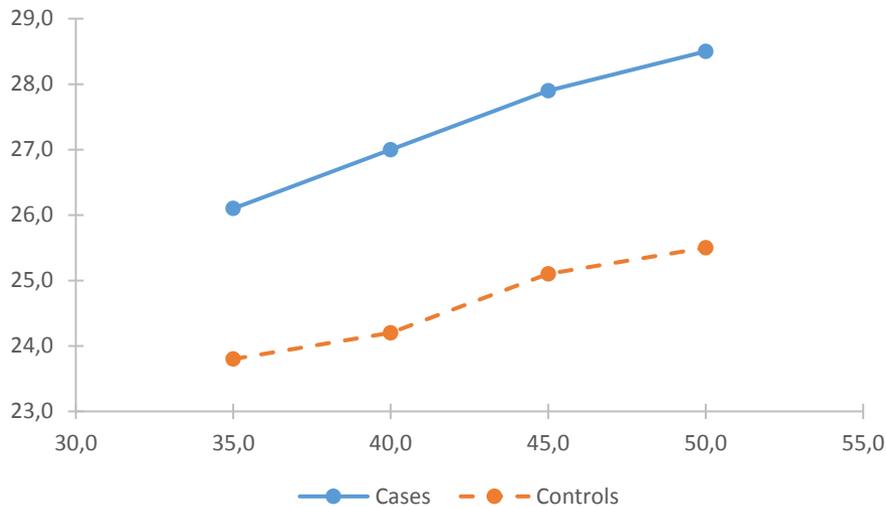


**Figure 5.** Means of SBP and DBP for HFE gene variants rs1799945 during follow-up time.

## 5.4 Periodic cohort health examinations

There were 339 cases and 604 controls in the study population. The study group was chosen from the PHE 50-year-old cohort ( $n = 6000$ ). Subjects having hypertension and/or diabetes and available data on 35-, 40-, and 45 year were chosen. In the group of cases 91 % had diagnosed hypertension, 15 % had diabetes and 9 % had both. Controls included healthy subjects from the same cohort and they were matched according to sex. Cases had already at the baseline at the age of 35 years old higher mean BMI, systolic and diastolic blood pressure and serum cholesterol than controls. Smoking habits and physical exercise were similar between the groups during the whole follow-up time. Cases used more alcohol during the follow-up time and their self-reported health status was poorer when compared with control group. At the baseline among cases there were 9 subjects who had hypertension diagnosis and 2 who had diabetes diagnosis already at the age of 35.

In the case group subjects gained weight during follow-up time on average 7.0 kg and controls 4.9 kg. BMI increased from 26.1 to 28.5 in the case group and from 23.8 to 25.5 in control group. The trend is shown in Figure 6.



**Figure 6.** BMI during 15 years of follow-up in case and control groups.

There was a statistically significant difference in BMI increase between study group and control group ( $p = 0.04$ ). BMI increased more in the study group and increase rate was constant. Also in the control group BMI increased, but the increase rate was slightly slower. Systolic blood pressure rise was statistically higher in the study group when compared with control group during the follow-up time ( $p = 0.004$ ). Increase rate in diastolic blood pressure did not differ between the groups markedly ( $p = 0.06$ ). Among cases at the age of 50 years 61 % were using blood pressure medication.

# 6 DISCUSSION

## 6.1 *STK39*

According to our results, rs6749447 in *STK39* gene had a significant effect on hypertension. In our study rs6749447 polymorphism in *STK39* gene, encoding serine/threonine kinase 39, was associated with hypertension. *STK39* rs6749447 G allele was associated with hypertension among 50-year-old Finnish men and women. A similar association has previously been found in systolic blood pressure among Amish population and Swedish women (Fava et al., 2011; Y. Wang et al., 2009).

Family history of hypertension and BMI were strong factors predicting hypertension in our study. Despite ethnic difference between study populations, the genotype frequencies were similar in our study and the study of Wang et al. (2009). BMI and BP are strongly associated (K. Liu et al., 1996; Mathieu et al., 2009). The effect of BMI on hypertension is discussed in literature review of this study and BMI needs to be considered when studying hypertension genetics. It has been approximated that 65-78 % of hypertension cases are attributed to obesity. (Rahmouni et al., 2005) The effect of increase of BMI by one unit was 0.6 mm Hg rise in DBP for women and 1.0 mm Hg for men, respectively (K. Liu et al., 1996). Also in our study obesity was strongly associated with hypertension. When our study population was stratified to different BMI classes the effect of *STK39* gene polymorphism was similar for average-weight and overweight subjects, which further confirms the role of *STK39*. Among obese (BMI > 30) subjects the effect of *STK39* was not significant, which reflects the strong effect of BMI on hypertension. Environmental factors affect gene expression strongly and therefore there are many factors responsible for BP regulation on the daily basis. (Marteau et al., 2004)

Renal-pressure natriuresis has a central role in long term blood pressure regulation (Hall et al., 2015). According to studies renal-pressure natriuresis is impaired in all forms of hypertension, also in essential hypertension. Overweight and obesity seem to be associated with impaired natriuresis. Excess weight gain impairs pressure natriuresis and increases renal tubular reabsorption. Prolonged obesity also causes structural changes in the kidneys. (Hall, 2003) This may also explain partly

why there was no association of *STK39* polymorphism with hypertension in obese subjects.

Kidneys have a major role in blood pressure control by regulating fluid and electrolyte balance (Cowley, 2006). *STK39* is part of a multigene kinase network, which regulates renal Na and K excretion. *STK39* affects NCC and NKCC2 cotransporters, which are major transport channels for salt reabsorption in kidneys. The kinase networks changes the mineralocorticoid hormone response, which affects sodium and potassium conservation and excretion. (Castañeda-Bueno & Gamba, 2010; Welling et al., 2010) Polymorphism in *STK39* gene may cause increased expression of *STK39*, which leads into increase in NCC phosphorylation. This promotes salt retention and results in blood pressure elevation. (Castañeda-Bueno & Gamba, 2010; Glover & O'Shaughnessy, 2011) Therefore *STK39* has been seen as a possible target for novel antihypertensive drug therapies (Glover & O'Shaughnessy, 2011).

Blood pressure response to antihypertensive drugs varies remarkably between individuals (Hiltunen & Kontula, 2012; Hiltunen et al., 2015). *STK39* gene polymorphism rs6749447 has been associated with BP response to antihypertensive drug losartan. Losartan is an angiotensin-II receptor blocker. Blood pressure decrease using losartan medication was 6.8 mm Hg for TT, 4.6 mm Hg for TG and 3.4 mm Hg for GG-carriers, respectively. Minor allele was associated with diminished response on blood pressure. (Donner et al., 2011) In our study there were 19 subjects having losartan medication, but further analyses could not be conducted, because they had also other medications combined with losartan. Anyhow the difference in drug response further confirms possible functional differences between individuals carrying one of the *STK39* gene variants.

## 6.2 SLC7A1

Endotelial NO formation is reduced in hypertension, which has been shown in studies comparing hypertensive and normotensive subjects. This may be due to environmental or genetic factors affecting L-arginine transport system, which alter the NO production significantly. (Linke et al., 2003; Yang & Kaye, 2006) Polymorphism rs41318021 in SLC7A1 gene, which encodes arginine transporter, has been associated with essential hypertension in previous studies (Yang & Kaye, 2006; Yang et al., 2007).

It has been shown that there is a difference in gene expression between *SLC7A1* gene polymorphism rs41318021 carriers and normal gene carriers. This was shown using reporter gene constructs. Minor allele T was associated with diminished gene expression when compared with C allele carriers. It was also found that allelic frequencies were statistically different between hypertensive and normotensive subjects in the study population, Australians. The frequencies of T allele were 13.3 % for hypertensive and 7.6 % for normotensive subjects, respectively. (Yang & Kaye, 2006; Yang & Kaye, 2009)

In the present study the frequencies of the *SLC7A1* gene polymorphism carriers and normal gene carriers were similar compared with previous study (Yang & Kaye, 2006). However the rs41318021 polymorphism was not associated with hypertension as it was in the earlier study (Yang & Kaye, 2006). Borderline effect of this variation was shown in our study among 35-year-old subjects, which is in line with the observation that among younger population the effect of a single polymorphism is more clearly shown (Munroe, Johnson, & Caulfield, 2009). According to GWAS-studies the effect of a single gene polymorphism has reported to be +1.16 mmHg/one allele at the highest (Munroe et al., 2009). This same magnitude was seen in our study. The gene effects are more likely to be seen among younger subjects, while among older subjects aging, overweight and other environmental and also gene-environmental interactions play a more significant role.

Endothelial function declines due to age and aging is associated with reduced vasodilatation of endothelium, which may explain the different results in the studies of L-arginine availability and hypertension. (Panza et al., 1993; Schlaich et al., 2004; Taddei et al., 1995)

### 6.3 *HFE*

In our study population the *HFE* gene was significantly associated with hypertension among 50-year old Finnish population. Association with hypertension was also seen in a previous GWAS study (Ehret et al., 2011). *HFE* gene studies have concentrated on hemochromatosis and we aimed to study the effects of possible mild iron overload on the risk of hypertension. An important finding was that subjects with at least one *HFE* 63D allele had higher BP already at the age of 35 years. Hypertension is a significant risk factor for heart disease, stroke and renal disease (Kearney et al., 2005) and therefore high blood pressure at younger age can lead to severe consequences already at younger age. It has been shown that even as small as 2 mm

Hg increase in systolic BP is involved with 10 % higher mortality on stroke and 7 % for other vascular causes. This may lead for example to ischemic heart disease already in middle age (Lewington et al., 2002). According to our study *HFE* gene polymorphism carriers had higher systolic and diastolic blood pressure at the age of 35 and 40. The difference compared with normal gene carriers was +4/2 mm Hg. At the age groups of 45 and 50 years the difference was no longer statistically significant. Difference of blood pressure variation after 45 years was difficult to study due to BP medication, since many of the hypertensive subjects already had medication in use. We tried to take into account the hypertension medication using modifying calculations, but the available data of medication was incomplete and results gained were inconsistent.

Obesity is a major risk factor for hypertension. This was clearly seen also in our studies. In our previous studies overweight and obesity has masked the genetic effect among groups of BMI>25 or 30, but the *HFE* H63D variant remained significant even when BMI was taken into account. Surprisingly the highest risk for hypertension was seen among obese subjects, OR was 4.2 for G-allele carriers. When overweight and obese subjects were analysed together, the effect was stronger than among normal weighed subjects. According to Zafon et al. (2010) obesity is associated with a deficit in serum iron levels and higher ferritin levels. Low iron levels in serum could be due to either true or functional iron deficiency. One possible explanation for increased ferritin levels could be inflammatory processes caused by obesity. see review by (Zafon, Lecube, & Simo, 2010)

Heterogeneity of blood pressure response to change in weight is called weight sensitivity. Kostis et al. (2013) found association between some polymorphisms related to hypertension, obesity and diabetes mellitus and weight sensitivity of BP. (Kostis et al., 2013) Overweight from adolescence compared with subjects becoming obese in adulthood seems to lead to higher risk for hypertension. Therefore length of time being overweight has a detrimental effect on blood pressure. On the other hand losing weight in adulthood was associated with similar risk of hypertension than subjects remaining normal weight from adolescence to adulthood. Also for all studied racial/ethnic groups being chronically overweight infused the risk of hypertension even more than gaining weight. (Suglia, Clark, & Gary-Webb, 2013)

Mild iron overload is a possible explanation for higher BP of H63D gene polymorphism carriers. The mechanism for iron overload caused by H63D is not fully understood. Iron absorption occurs into enterocytes in the duodenum and exportation into circulation is due to ferroportin. Iron is bound to transferrin in circulation. (Means Jr, 2013) The role of H63D in the iron metabolism occurs via

hepcidin, which is an iron regulatory hormone (Ganz, 2011; Muckenthaler et al., 2017). Under normal physiologic circumstances iron excess stimulates hepcidin production. Hepcidin acts as a feedback inhibitor and gene polymorphism in *HFE* gene affects the regulation of iron absorption by blocking hepcidin. Therefore *HFE* gene polymorphism may lead to iron accumulation in the body. (Canavesi et al., 2012; Ganz, 2011; Zacharski, 2010) The blood pressure levels have reported to be higher among hemochromatosis patients compared with controls. The difference was remarkable, 10 mm Hg in systolic and 8 mm Hg in diastolic blood pressure. Possible explanation for difference is iron overload.(Cash et al., 2014)

The studies of H63D gene polymorphism report controversial results of iron overload. In a large meta-analysis H63D polymorphism was associated with iron overload among homozygotes, but not among heterozygotes (Neghina & Anghel, 2011). It has been suggested that additional modifying factors are needed and H63D polymorphism itself is insufficient to cause significant iron overload. (Gochee et al., 2002) According to Samarasena et al. (2006) *HFE* D63D homozygosity was associated with elevated ferritin levels. Also transferrin saturation levels were elevated. (Samarasena et al., 2006) *HFE* D63D homozygotes may have severe or mild iron overload or no iron overload at all. The genetic modifiers in the *HFE* or *TfR* genes may have a remarkable effect, but their role is not fully understood. (Aguilar-Martinez et al., 2001; Pietrangelo, 2016) Serum ferritin and transferrin saturation levels could be measured from blood samples to assess body iron levels reflecting iron overload. (Neghina & Anghel, 2011) These saturation levels were not included in the TAMRISK data and we were not able to observe iron overload levels of subjects. In previous studies men with essential hypertension had elevated iron stores compared with normotensives (Piperino et al., 2002).

## 6.4 Periodic health examinations

In our study the effect of the PHEs was not strong enough to stop weight gain among either of the groups. The case group consisted of subjects who were hypertensive by the age of 50 years. There was a statistically remarkable difference between the BMI increase in the case group and control group during the follow-up time. In the case group the increase was constant while in the control group the rate of increase slowed slightly during the last 5 years of follow-up time.

At the beginning of the follow-up the subjects in the case group had higher BMI already by the age of 35. Obesity is known to be a major risk factor for cardiovascular diseases and diabetes and therefore case group subjects had a greater risk already in the beginning. According to this the most effective preventive actions could have been allocated especially to case group instead of control group in the PHEs. According to our results the PHE could not prevent further weight gain among subjects having high risk for cardiovascular diseases and diabetes.

Blood pressure rise was observed according to the increase of BMI. Both systolic and diastolic blood pressures were higher among case group than among controls in the beginning. Also the increase was steeper in the case group between ages 40 and 45 years. The effects of PHEs were not shown in blood pressure even though over half of the subjects had received blood pressure medication during the follow-up period.

Participants' opinions and personal experiences of the PHEs at the age of 45 years old have previously been studied (Nupponen, 1996). The discussions and counselling were arranged with a public health care nurse. Counselling dealt mainly with living habits, also for some extend with symptoms, diseases and prevention of diseases. PHEs were seen beneficial by the respondents. When benefits of PHEs were compared with medical assistance of a physician, the opinions varied. According to our study the PHEs, when conducted at 5 years intervals, were not very effective in changing living habits. It has been shown that overweight subjects, BMI over 25.0 but below 29.9, have significantly increased risk of diabetes, hypertension, heart disease, stroke and gallstones (Field et al., 2001). The case group subjects had hypertension, diagnosed by a physician, but the regular intervention and medication were clearly insufficient, larger proportion of cases would have needed hypertension medication. Among Finnish adult 25-64 years old population the prevalence of hypertension (systolic blood pressure over 140 mm Hg) is estimated to be over 48 %, which is among the highest in Europe (Geleijnse et al., 2004)

When the alcohol consumption was compared between the case group and control group at the age of 35 years, the difference was remarkable. Subjects of case group consumed 95 g of alcohol per week and controls 79 g, respectively. Amounts were reported by subjects themselves and therefore absolute amounts are presumably larger. In Finland the mean consumption of alcohol was 9.7 l per capita during years 2002-2006 (Westman et al., 2015). This corresponds to 147 g per week. Physical activity was similar in the study group and in the control group. Therefore difference in alcohol intake is one possible explanation for increase in BMI in the case group.

## 7 CONCLUSIONS

The major findings and conclusions are:

### I

Our study demonstrates that *STK39* polymorphism rs6749447 is associated with hypertension among 50-year-old Finnish subjects. This further supports the role of *STK39* as a susceptibility gene for hypertension. Gene polymorphisms associated with hypertension may also have remarkable effect on the drug response and therefore knowing genetic background could enhance the drug therapy response among hypertensives in the future.

### II

The effect of gene variation rs41318021 in gene *SLC7A1* on hypertension was of borderline significance among 35 years old subjects and not shown among 50-year-old subjects possibly due to environmental factors.

### III

Our study suggests that minor D-allele of the H63D variant rs1799945 is associated with higher risk for hypertension at the age of 50 years when compared with normal gene carriers. The mechanism is not yet known. We also found that gene polymorphism carriers had higher BP already at the age of 35 years. The difference in systolic and diastolic BP was relatively small, but the long-term consequences may be remarkable. Further studies with iron measurements are needed to prove the relationship between H63D variation, mild iron overload and hypertension.

### IV

Our results suggest that there were no apparent favourable effect on lifestyle factors by PHEs among the subjects in the case group of subjects with hypertension at the age of 50 years. They had higher risk already at the age of 35 years. The difference

was already seen at the age of 35 years in the beginning and there occurred no correction during the 15 years of PHE period.

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*Kirsi Määttä*

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## 10 ORIGINAL PUBLICATIONS

# A functional variant in the serine-threonine kinase coding gene is associated with hypertension: a case–control study in a Finnish population, the Tampere adult population cardiovascular risk study

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**Objectives:** Hypertension raises the risk of cardiovascular consequences to two-fold or three-fold. The incidence of hypertension is increasing worldwide. Genetic causes of blood pressure are estimated to cause half of the hypertension effect, but the genes behind this are still fairly unclear. Polymorphisms in gene STK39 (serine/threonine kinase) have in some studies been associated with hypertension, but results have differed according to genetic population. We screened the STK39 polymorphism rs6749447 in a Finnish cohort to see if it was associated with hypertension.

**Methods:** The study included 447 hypertensive cases and 771 controls. All participants were 50-year-old Finnish patients and the data was collected from the Tampere adult population cardiovascular risk study (TAMRISK). Genotypes were determined by polymerase chain reaction using DNAs extracted from buccal swabs.

**Results:** The risk for hypertension among G-allele carriers was 1.4-fold compared with controls ( $P=0.006$ , 95% CI = 1.10–1.79). The genetic effect of the G-allele was even more significant when the strong effect of BMI on hypertension was taken into account: for normal weight patients (BMI < 25) the risk was two-fold ( $P=0.003$ , 95% CI 1.3–3.1) and for normal weight or slightly overweight patients (BMI < 30), the risk was 1.6-fold ( $P=0.001$ , 95% CI 1.2–2.2).

**Conclusion:** In conclusion, there was a significant association between STK39 genetic variant rs6749447 and hypertension in a Finnish cohort.

**Keywords:** genetic variants, hypertension, serine-threonine kinase coding gene

**Abbreviations:** BP, blood pressure; CI, confidence interval; GWAS, genome-wide association study; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OR, odds ratio; PHE, periodic health examination; SNP, single nucleotide polymorphism; STK39, serine-threonine kinase coding gene; TAMRISK, Tampere adult population cardiovascular risk study

## INTRODUCTION

It is estimated that from year 2000 to 2055 hypertension incidence rises from 26.4 to 29.2% worldwide [1]. Essential hypertension is a significant risk factor for heart disease, stroke and renal disease. High blood pressure (BP) causes more than a third of deaths in Europe and contributes to about half of the risk for ischemic heart disease and stroke [2,3]. The risk of cardiovascular consequences is increased to two-fold or three-fold by hypertension [4].

Twin studies have revealed that even over 50% of differences in BP are genetically determined [5–7]. The inheritance of BP is rather complex containing many possible genes, although most of them are still unknown [8]. Genome-wide association studies (GWAS) have made it possible to dissect hundreds of thousands single nucleotide polymorphisms (SNPs) in one chip, although the results have not been as effective as thought. The drawback of GWAS studies is their poor reproducibility due to differences between populations, matching of cases and controls, stratification in populations and heterogeneity in alleles and genes [8,9]. Until year 2010, eight SBP, 11 DBP and six hypertension genes have been identified using GWAS [10]. All these single genes encode molecules that affect salt balance regulated by kidneys [11].

Serine-threonine kinase coding gene (STK39) gene that encodes serine/threonine kinase 39 has been identified as a possible candidate gene associating with primary hypertension [12–14]. In a GWAS, Wang *et al.* [12] found that rs6749447, located in intron 1 of the STK39 gene, associated significantly with hypertension. They also showed that different gene variants in STK39 gene might have an

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influence on BP by increasing STK39 expression, which leads to altering of renal sodium excretion. It was also observed that the protein structure of STK39 was not altered. The study was conducted in an Amish population, which is seen as a homogenous lifestyle living population and well suitable for identification of genes of complex diseases.

In addition to rs6749447, the other SNPs in the STK39 gene that associate with hypertension are rs3754777 and rs6433027 in men [12,13], and rs35929607 in women [14]. The rs35929607 polymorphism was associated with approximately 21% increase in hypertension prevalence in women [14]. However, Cunningham *et al.* [15] found no such association for rs6749447, rs3754777 and rs35929607 in white British.

When hypertension occurs after the age of 70 years, the family history of hypertension is not remarkable, but in cases below age of 60 years family history becomes more important. Therefore, hypertension scans for patients over 60 years old reveal less clearly the genetic component of hypertension [9].

The aim of this study was to test the association between polymorphism of the STK39 rs6749447 and hypertension among 50-year-old Finnish patients.

## METHODS

### Participants

The Tampere adult population cardiovascular risk study (TAMRISK) is a prospective, longitudinal population-based health survey study in Tampere, a city in southern Finland with a population of 210 000. The Tampere city healthcare centre has provided regular periodic health examinations (PHE), for screening and counseling, for the adult population of the city since 1980. All 40 and 50-year-old inhabitants have been invited to participate in these health surveys. During some periods, also 35 and 45-year-olds have been invited to participate. The PHE consists of one 60-min session with a public health nurse at the centre's health examination unit. A standard questionnaire on subjective health and health behavior and referral to basic laboratory tests are sent with the invitation letter. In the session, the questionnaire information and screening tests are reviewed. Counseling is given on topics selected by the client and also on findings of the screening tests. Informed consent was obtained at the time of the physical examination for use of the data for research.

The data for the TAMRISK study was collected from the PHE done for 50-year-old men and women living in Tampere. TAMRISK data includes information of risk factors for hypertension: BP, weight, family history of cardiovascular diseases, lipid values and smoking, diabetes and exercise habits. Buccal swabs for DNA extraction and a permission form to use PHE data were collected by mail separately of the physical examination. The DNA samples were collected during years 2006–2010. Informed consent was obtained from all participants. The Ethics Committees of the Tampere University Hospital and the City of Tampere approved the study.

Cases ( $n = 447$ ) in this study were the patients who had hypertension at the age of 50 (as diagnosed by a physician)

and for each case, at least one normotensive control ( $n = 771$ ) with the same sex and similar smoking habits, were chosen from a PHE cohort ( $n = 6000$ ). Smoking status was evaluated based on self-reporting. Finally, we selected a subpopulation of men and women who had available data of hypertension measurements at the age of 35, 40, 45 and 50 years.

### DNA genotyping

DNA was extracted from buccal swabs using a commercial kit (Qiagen Inc., Valencia, California, USA). Genotyping was performed using designed different allele-specific primers: 5'-CCTGTATTTACAAGCCCC ACA-3' as a right primer and either 5'-AGTCTGCTAGTACTAGATTAGGAG-3' or 5'-GAGTCTGCTAGTACT AGATTAGGAT-3' as a left primer. The difference between left primers was their last nucleotide in the 3' end: G/T. Two parallel polymerase chain reactions (PCRs) were performed for each sample. Amplification was done in 94°C for 15 min that followed 34 cycles of 94, 55 and 72°C for 30 s each. The final extension was at 72°C for 5 min. The PCR products were run in 1% agarose gel electrophoresis.

### Statistical analysis

Analysis of variance for continuous variables and  $\chi^2$  test for categorical variables were applied for the comparison according to cases and controls at the age of 50 years. If the distribution was skewed, the analysis was performed using transformed values to approximately normalize the distribution. To assess the effect of STK39 on the risk of hypertension, we used binary logistic (Forward Wald) regression analysis. The analysis of variance for repeated measures was used to assess the differences in mean BPs between genotypes at the age of 35, 40, 45 and 50 years. The model included the main effects of group factor and time, and their interaction.  $P$  values less than 0.05 were considered significant. Analyses were carried out using SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

## RESULTS

Clinical characteristics of the cases (447) and controls (771) are presented in Table 1. In the case group 7.9% of men and 5.4% of women had diabetes. The control group was apparently healthy, although BMI and cholesterol values were above the national recommendations.

In the whole study population the frequencies of the STK39 gene variants were 0.53 for TT ( $n = 636$ ), 0.46 for TG ( $n = 546$ ) and 0.02 for GG ( $n = 18$ ). Genotype frequencies of the cases with confirmed hypertension were compared with controls. In the hypertension group, frequencies were 0.48 for TT ( $n = 192$ ), 0.51 for GT ( $n = 204$ ) and 0.02 for GG ( $n = 8$ ), whereas in the control group they were 0.56 for TT ( $n = 420$ ), 0.43 for GT ( $n = 324$ ) and 0.01 for GG ( $n = 10$ ). As there were only 18 patients who had genotype GG, they were combined to GT group for further analysis. Using  $\chi^2$  test, genotype frequencies differed significantly between cases and controls ( $P = 0.026$ ).

To find the interpretative factors for hypertension we used logistic regression analysis. When the association of STK39 was defined alone the risk for hypertension among

**TABLE 1. Clinical characteristics of cases and controls of the study**

Clinical characteristic	Cases (n = 447)	Controls (n = 771)	P
Age (years)	50 ± 0	50 ± 0	
BMI (kg/m <sup>2</sup> )	28.6 ± 5.1	25.4 ± 3.6	<0.001
Cholesterol (mmol/l)	5.4 ± 1.0	5.4 ± 1.0	0.7
HDL cholesterol (mmol/l)	1.6 ± 0.4	1.7 ± 0.4	0.4
LDL cholesterol (mmol/l)	3.2 ± 0.9	3.2 ± 0.8	0.9
Triglycerides (mmol/l)	1.5 ± 1.2	1.2 ± 0.7	0.03
Glucose (mmol/l)	5.1 ± 1.2	4.8 ± 0.6	>0.05
SBP (mmHg)	143 ± 16	130 ± 15	<0.001
DBP (mmHg)	93 ± 9	84 ± 9	<0.001
Hypertension (%)	100	0	<0.001
Exercise (at least twice a week) (%)	66	72	0.06
Diabetes (%)	7	0	<0.001
Lipid-lowering drugs (%)	15	3	<0.001
Myocardial infarction (%)	2	0	<0.001
Blood pressure medication (%)	67	0	<0.001
Sex (male) (%)	60	57	

Data are presented as mean ± SD.

G-allele carriers was 1.4-fold compared with controls ( $P=0.006$ , CI = 1.10–1.79).

Because the association of BMI on hypertension was expectedly strong ( $P<0.001$ ), the study population was divided according to BMI to four groups using WHO classification (Table 2). When STK39 gene, BMI class, family history of hypertension and sex were included as explainable variables in logistic regression stepwise analysis (Forward Wald), STK39 increased the risk to hypertension 1.5-fold, almost the same as for slightly overweight (1.7-fold). Family history of hypertension increased the risk to 3.5-fold (Table 2).

The association of STK39 gene variants with hypertension was further calculated separately for those who were normal weight (BMI < 25 kg/m<sup>2</sup>) and for normal weight or slightly overweight (BMI < 30 kg/m<sup>2</sup>). For patients with normal weight the association of STK39 gene was even more significant (OR = 2.0,  $P=0.003$ , 95% CI 1.3–3.1), as was the case also for those with normal or slight overweight (OR = 1.6,  $P=0.001$ , 95% CI 1.2–2.2). When BMI was over 30 kg/m<sup>2</sup> the effect of STK39 was no longer significant ( $P=0.832$ , 95% CI 0.6–1.9). These results indicate that in addition to BMI, also STK39 polymorphism had a significant effect on hypertension.

Finally, we selected a subpopulation of men and women who had available data of hypertension measurements at the age of 35, 40, 45 and 50 years. This follow-up data was available from 780 participants. Repeated measures analysis showed that those with the minor rs6749447 G-allele

( $n=362$ ) had higher SBP and DBP during the whole 15 years follow-up period ( $P=0.06$  for SBP,  $P=0.016$  for DBP) compared with major allele homozygotes ( $n=418$ ) (Fig. 1). However, no statistically significant interaction was found in the change of BP between the G-allele carriers compared with major allele homozygotes, and the follow-up time.

## DISCUSSION

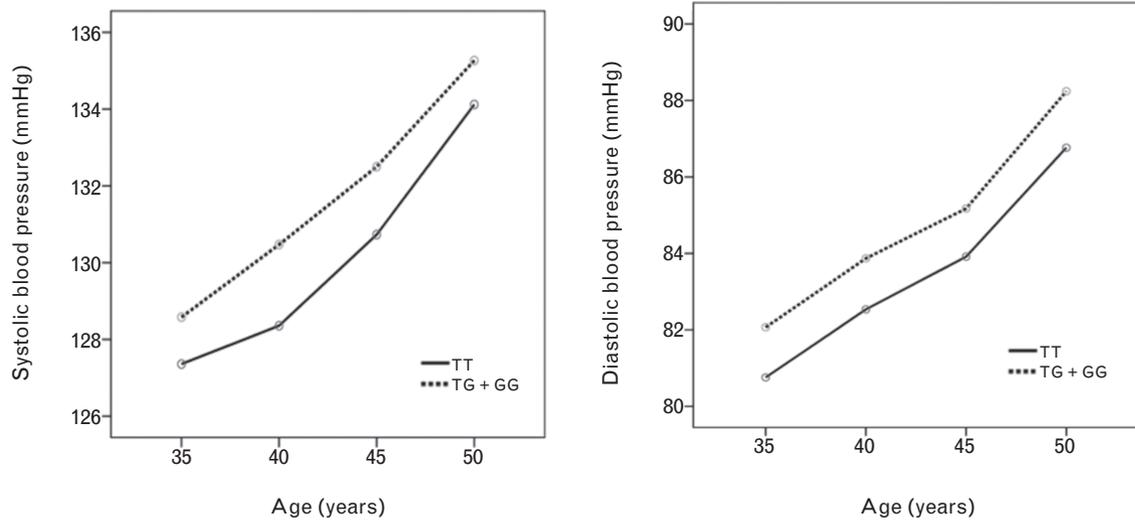
STK39 gene that encodes serine/threonine kinase 39 is identified as a possible candidate gene associating with primary hypertension [12,14]. The present study suggests that the G-allele of the genetic variant in the STK39 (rs6749447) gene is associated significantly with hypertension among 50-year-old Finnish men and women. This is in line with the study by Wang *et al.* [12] who found strong evidence of association between SBP and gene polymorphism at the same genetic site ( $n=790$ ). In addition, we also found that family history of hypertension and especially BMI were strong factors behind hypertension in our study population. The genotype frequencies in our study population were similar to the study of Wang *et al.* [12] even though there was an ethnic difference in the study populations.

BMI is an important factor to take into consideration when studying hypertension, because BMI and BP are very strongly associated [16,17]. In obesity, sympathetic nervous system and renin–angiotensin system are activated. Also

**TABLE 2. The effect of statistically significant factors on hypertension incidence**

Factor	OR	P	95% CI
rs6749774-G allele	1.5	0.003	1.16–2.00
BMI: BMI <25 kg/m <sup>2</sup>	1.0		
BMI 25–29.9 kg/m <sup>2</sup>	1.7	0.001	1.23–2.27
BMI 30–34.9 kg/m <sup>2</sup>	5.2	<0.001	3.44–7.76
BMI >35 kg/m <sup>2</sup>	13.6	<0.001	6.69–27.55
Family history of hypertension	3.5	<0.001	2.65–4.66

Analyzed with logistic regression stepwise analysis (Forward Wald). The model included classified BMI, gender, rs6749774 gene variation, and family history of hypertension. Chi-square 209.229,  $P<0.001$ ,  $r^2=0.171$  (Cox & Snell).



**FIGURE 1** Means of SBP ( $P=0.06$ ) and DBP ( $P=0.016$ ) in different ages according to STK39 genetic variants.

aldosterone levels increase in plasma. In consequence, abnormal sodium retention occurs and arterial pressure rises [18]. It has been reviewed that 65–78% of hypertension cases might be attributed to obesity [17]. In the study of Liu *et al.* [16], an increase of BMI by one unit increased DBP 0.6 mmHg for women and 1.0 mmHg for men. In the present study, we also found a strong association between obesity and hypertension. However, after stratifying the study population to different BMI classes, the results were similar for normal weight and slightly overweight patients, confirming the role of STK39. When obese patients ( $BMI > 30 \text{ kg/m}^2$ ) were analyzed in their own group, the effect of STK39 genotype was no more significant. This may indicate that obesity will override the effect of STK39. There are many environmental factors that affect the gene expression and which are responsible for the BP regulation on the daily basis. The gene may express its effect differently depending on its environment. In fact it has been shown that association of hypertension gene (SELE 554L/F) is also in relation to BMI [19].

Kidneys have a major role in the regulation of fluid and electrolyte balance, and therefore in determining BP [20]. A multigene kinase network regulates renal  $\text{Na}^+$  and  $\text{K}^+$  excretion. STK39 is part of this network. This protein affects  $\text{Na}^+:\text{Cl}^-$  and  $\text{Na}^+:\text{K}^+:2\text{Cl}^-$  cotransporters (NCC and NKCC2) in kidneys where they are the major transport channels for salt reabsorption [11,21,22]. The kinase network alters the response of kidneys to mineralocorticoid hormone response, which regulates the sodium and potassium conservation and excretion, respectively [11,21,22]. SNPs may cause increased expression of STK39 in the kidneys, which may increase NCC phosphorylation and promote salt retention. As a result, BP rises [21,22]. Considering these effects, STK39 seems to be a possible target for novel antihypertensive drug therapies [12,22].

The response of BP to antihypertensive drugs is suspected to have a remarkable variance between individuals [8]. Recently, Donner *et al.* [23] have reported that rs6749447 in the STK39 gene is the only variant out of the 19 selected SNPs that associated with BP response to

antihypertensive drug losartan. The minor allele (G) was associated with diminished response. Observed BP decrease with losartan medication was 3.4 mmHg for GG-, 4.6 mmHg for TG- and 6.8 mmHg for TT-carriers, respectively. Losartan is an angiotensin-II receptor type 1 blocker [23]. This observation further confirms possible functional differences between individuals carrying different STK39 genetic variants. In the present study, there were only 19 patients who had losartan medication. As this drug was also combined with other BP medications, we were unable to verify the above finding.

In conclusion, our study demonstrates an association between rs 6749447 variants and hypertension among 50-year-old Finnish patients supporting further the role of STK39 as a susceptibility gene for hypertension.

### Study strengths and limitations

All patients participating in the study were 50 years old when the PHE clinical examination took place. Available follow-up data of hypertension measurements were also available for a subpopulation at the age of 35, 40, 45 and 50 years. Part of the TAMRISK data bases on the patient's own reporting, which may cause some bias to the results. However, the most relevant data for our study was measured data, for example BP. And even if there was some bias in the measurements, there is no net effect due to that. Because the study was done for a Finnish cohort, our results cannot be generalized for populations having different genetic background.

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### Conflicts of interest

There are no conflicts of interest.

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## Reviewers' Summary Evaluations

### Referee 1

The authors examine the hypothesis that the STK39 SNP rs6749447 is associated with hypertension among Finnish subjects from the TAMRISK study. Strengths of the article include: a clearly stated hypothesis, a reasonable study design and well presented analysis. Other strengths are the findings of the modulation of the genetic risk by BMI and the additional evidence of the effect of the SNP on BP from a longitudinal study. Weaknesses are a modest sample

size and the potential lack of generalizability to other populations with different ancestry.

### Referee 2

The work shows good methodology and attention to relevant detail. The findings are well presented and should stimulate some interest within the field. The authors acknowledge that there are weaknesses due to the numbers studied and that the subjects are all from a restricted genetic pool so may not extrapolate to different genetic populations. Nevertheless this is a well designed and presented study.

RESEARCH ARTICLE

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# Contribution of SLC7A1 genetic variant to hypertension, the TAMRISK study

Kirsi Määttä, Tarja Kunnas\* and Seppo T Nikkari

## Abstract

**Background:** The rs41318021 polymorphism in the SLC7A1 gene affects endothelial NO production through changes in L-arginine transport. This variation could thus hypothetically cause dysfunction of endothelium and lead to hypertension. The association of rs41318021 with hypertension was therefore studied in a Finnish cohort.

**Methods:** A total of 412 hypertensive cases and 771 non-hypertensive controls from a Finnish 50-year-old cohort were included in this study. The data was collected from the Tampere adult population cardiovascular risk study (TAMRISK). DNA was extracted from buccal swabs and amplified using PCR. A subpopulation of men and women who had available follow-up data of blood pressure measurements at the age of 35-, 40-, 45- and 50 years was also analyzed.

**Results:** There was no difference between the variant frequencies of the hypertension group and normotensive group at the age of 50 years ( $p = 0.209$ ). However, repeated measures analysis from the 15-year follow-up showed that subjects having gene variants CT or TT had slightly higher diastolic blood pressure than subjects having genotype CC ( $p = 0.047$ ). By post-hoc analysis, this was most pronounced at the age of 35 years ( $p = 0.044$ ).

**Conclusion:** The rs41318021 polymorphism in the SLC7A1 gene was not associated with essential hypertension in 50-year-old subjects. However, a borderline effect of this variation upon diastolic blood pressure was seen in these same subjects in a 15-year follow-up from a 35-year-old cohort to 50 years of age.

## Background

Hypertension is a disorder caused by a complex combination of genetic and lifestyle risk factors. Hypertension raises the risk of incident cardiovascular disease to two- or three-fold [1]. The major contributors to hypertension in Western countries are overweight, physical inactivity, high salt intake and low potassium intake [2]. The genetic component in hypertension is estimated as 30 – 50% of the total impact [3]. Candidate-gene and genome-wide association studies (GWAS), have previously identified numerous genetic loci that are associated with blood pressure, but collectively these explain only a few percent of the heritability for hypertension [4,5].

The role of vascular endothelium is significant in the regulation of blood pressure. Endothelial cells synthesize vasodilatory factors like nitric oxide (NO). Arginine is the rate limiting substrate for endothelial nitric oxide synthase (eNOS), which acts as a catalyst in NO production [6-8].

SLC7A1 (previously CAT-1), located in chromosome 13q12-q14, encodes a cationic amino acid transporter for arginine and lysine uptake in mammalian cells [9,10]. Functional variation of SLC7A1 gene alters the expression of SLC7A1, which may result in changes of NO production and endothelial function [10]. Therefore, changes in SLC7A1 gene could cause dysfunction of endothelium and lead to hypertension.

To our knowledge, there is only one previous study that has addressed the association of rs41318021 with hypertension in an Australian population [10]. We wanted to assess the role of this variant in a Finnish population, by analyzing cohorts from the Tampere adult population cardiovascular risk study (TAMRISK).

## Methods

### Subjects

The data for the TAMRISK study was collected from periodic health examinations (PHE) done for 50-year-old men and women living in Tampere, Finland. TAMRISK data includes information of risk factors for hypertension:

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blood pressure, weight, family history of cardiovascular diseases, lipid values and smoking, diabetes and exercise habits [11]. Buccal swabs for DNA extraction and a permissions form to use PHE data were collected by mail separately of the physical examination. The DNA samples were collected during years 2006–2010. Informed consent was obtained from all participants. The Ethics Committees of the Tampere University Hospital and the City of Tampere approved the study.

Cases (n = 412) in this study were the subjects who had hypertension at the age of 50 years (as diagnosed by a physician) and for each case, at least one normotensive control (n = 771) with the same sex and similar smoking habits, were chosen from a PHE cohort (n = 6000). Smoking status was evaluated based on self-reporting. Finally, we selected a subpopulation of men and women who had available data of blood pressure measurements at the age of 35-, 40-, 45- and 50 years.

#### Baseline measurements

The basic evaluation in 1988–91 included an interview by a public health nurse. The interview was conducted using a structured questionnaire about health and health-related behaviour, including questions about current and previous diseases. Information on current and previous diseases was based on self-report of diagnosis by a physician, including history of coronary artery disease, myocardial infarction and diabetes. Family history of hypertension in a close relative was also asked in the questionnaire. The frequency of physical exercise comprised both leisure and commute related activity. Physical examination included a single blood pressure (BP) measurement (mm of mercury) using a calibrated mercury sphygmomanometer. Serum total cholesterol (mmoles/litre), glucose (mmoles/litre) and haemoglobin (grams/litre) were measured after an overnight fast by standard techniques. Height (cm) and weight (kg) were recorded from which the body mass index was calculated.

#### Genotyping

DNA was extracted from buccal swabs using a commercial kit (Qiagen Inc., Valencia, Calif., USA). Genotyping was performed using allele specific primers: SLC7A (047) 5'-AGT TGT CTG GAG GTG ACC-3' and SLC7A (050 T) 5'-GCAAGTGACGCACAGCCT-3' or SLC7A (049C) 5'-GCAAGTGAGGCACAGCCC-3' as described by Yang et al. (2007) [10]. Two parallel PCRs were performed for each sample. Amplification was done in 94°C for 15 minutes followed by 32 cycles of 94°C, 55°C and 72°C for 30 s each. The final extension was at 72°C for 5 min. The PCR products were resolved in 2% agarose gels.

#### Statistical analysis

Analysis of variance (ANOVA) for continuous variables and Chi-square test for categorical variables were applied for the comparison of cases and controls at the age of 50 years. If the distribution was skewed, the analysis was performed using transformed values to approximately normalize the distribution. To assess the effect of risk factors for hypertension, we used binary logistic (forwald wald) regression analysis. The analysis of variance for repeated measures was used to assess the differences in mean blood pressures between genotypes at the age of 35-, 40-, 45- and 50 years. This follow-up data was available from 775 participants.

P values less than 0.05 were considered significant. Analyses were carried out using SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

#### Results

Clinical characteristics of the cases (412) and controls (771) at the age of 50 years are presented in Table 1. The case group of hypertensive subjects was compared to healthy controls. Cases had higher body mass index (BMI), haemoglobin (Hb), triglycerides, fasting glucose, systolic and diastolic blood pressure, and lower HDL cholesterol compared to controls. Cases exercised more than controls and had higher frequency of diabetes, myocardial infarction and family history of hypertension.

In the whole study population the frequencies of the rs41318021 variants for SLC7A1 were 0.70 for CC (n = 828), 0.27 for CT (n = 319) and 0.03 (n = 36) for TT. There was no difference between the variant frequencies of the hypertension group and normotensive group (p = 0.209). In the hypertension group the frequencies were 0.69 for CC, 0.29 for CT and 0.02 for TT. In the control group these proportions were 0.71, 0.26 and 0.03, respectively.

To find the explainable factors for hypertension in the 50-year-old cohort we analyzed the data using logistic regression analysis. When SLC7A1 genotype, BMI, Hb, HDL cholesterol, triglycerides, glucose, and family history of hypertension were included in stepwise analysis (forward wald), BMI, (p < 0.001, OR: 1.19, 95% CI 1.14-1.24), glucose (p = 0.009, OR: 1.37, 95% CI: 1.08-1.75) and family history of hypertension (p < 0.001, OR: 3.28, 95% CI: 2.35-4.56) were associated with hypertension.

Finally we selected a subpopulation of men and women in the present study who had available data of blood pressure measurements at the age of 35-, 40-, 45- and 50 years. This follow-up data was available from 775 participants, with subjects from both the hypertension and control groups. Subjects with CT and TT genotypes were combined, because there were only 23 subjects in the TT group. Repeated measures analysis showed that subjects having gene variants CT or TT had slightly higher diastolic blood pressure than subjects having genotype CC

**Table 1 Clinical characteristics of study population at the age of 50 years**

	Cases (n = 412)	Controls (n = 771)	P
Age (years)	50 ± 0	50 ± 0	
BMI (kg/m <sup>2</sup> )	28.6 ± 5.1	25.4 ± 3.6	<0.001
Haemoglobin (g/l)	147.2 ± 13.2	143.90 ± 13.6	<0.001
Cholesterol (mmol/l)	5.40 ± 0.97	5.40 ± 0.92	0.965
HDL cholesterol (mmol/l)	1.56 ± 0.45	1.67 ± 0.44	<0.001
LDL cholesterol (mmol/l)	3.17 ± 0.87	3.21 ± 0.84	0.512
Triglycerides (mmol/l)	1.55 ± 1.20	1.17 ± 0.68	<0.001
Glucose (mmol/l)	5.14 ± 1.20	4.84 ± 0.55	<0.000
Systolic blood pressure (mm Hg)	142.93 ± 16.17	129.75 ± 14.66	<0.000
Diastolic blood pressure (mm Hg)	92.92 ± 8.95	84.44 ± 9.40	<0.000
Exercise (at least twice a week) %	33.8	28.0	0.048
Diabetes %	11.8	0	<0.000
Myocardial infarction %	2.5	0	<0.000
Family history of hypertension %	71.3	42.2	<0.000
Current smoking %	19	19	
Gender (male) %	58.4	56.8	0.319

Data are presented as mean ± SD.

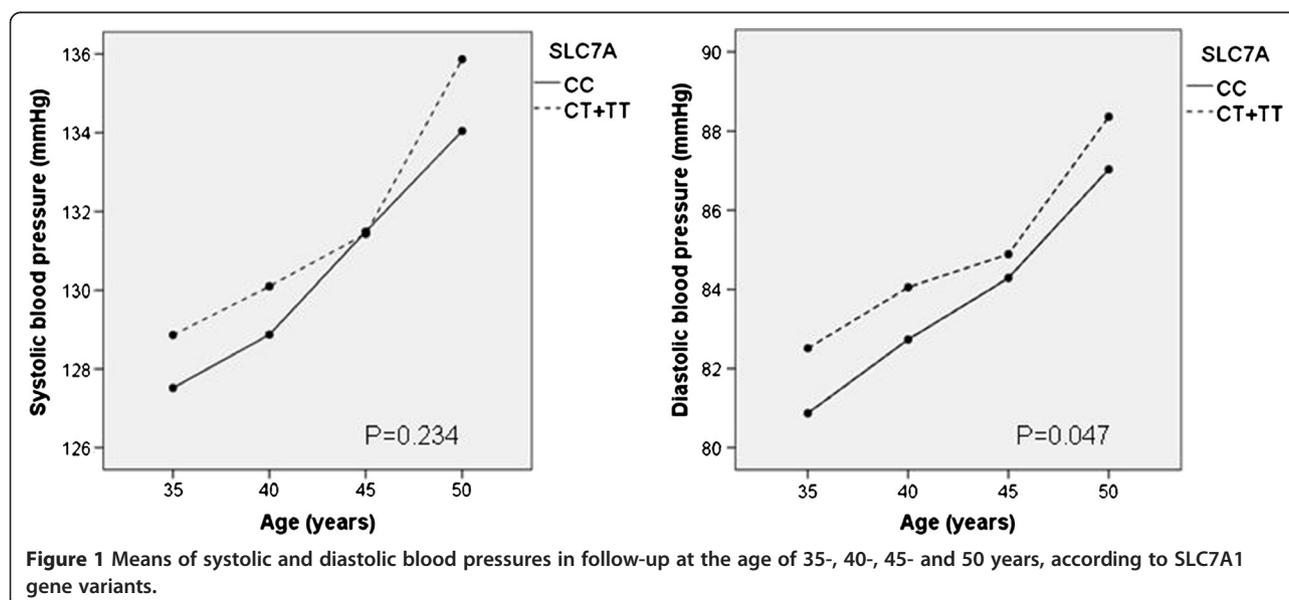
( $p = 0.047$ , Figure 1). The difference between genotypes in diastolic blood pressure was 1.4 mmHg at its highest at the age of 35 years. The trend was similar for systolic blood pressure, but not statistically significant ( $p = 0.234$ ). By one way ANOVA, the only statistical difference for diastolic and systolic blood pressure between the genotypes was for diastolic blood pressure at the age of 35 years ( $p = 0.044$ ).

When covariates that emerged as being significant in the logistic regression at the age of 50 years (BMI and glucose) were added to the repeated measures model,

the results remained the same in the follow-up. Subjects having gene variants CT or TT had higher diastolic blood pressure than subjects having genotype CC ( $p = 0.021$ ). Again, the trend for systolic blood pressure was not statistically significant ( $p = 0.241$ ).

## Discussion

Gene variation rs41318021 in the human SLC7A1, which encodes the principal arginine transporter, has previously been proposed to associate with a genetic predisposition to essential hypertension [10,12]. Our study subjects were



from Finland, where it is estimated that over 48% of 25–64 year old adults have mean systolic pressure of 140 mmHg or above, which is among the highest in Europe [2]. The association of rs41318021 with hypertension was therefore studied in this Finnish cohort.

Environmental and genetic changes in L-arginine transport are known to influence NO production significantly. Endothelial NO formation is reduced in subjects with essential hypertension, when compared to normotensive subjects [12]. Synthesis of NO requires substrate L-arginine [13]. Vasodilatation may be affected by limitation of substrate (L-arginine) for the synthesis of NO. The actual effect of decreased availability of L-arginine on impaired endothelial function is still controversial. Increasing age is associated with declining endothelial function [14], which might explain some of the differences between contradictory results on L-arginine availability and hypertension [15,16].

L-arginine transporter SLC7A1 (previously CAT-1) has an important role in L-arginine availability [8]. Yang and Kaye [17] were able to show differences in gene expression between alleles of the SLC7A1 gene (rs41318021). Using reporter gene constructs they showed that minor allele T in rs41318021 is associated with lower gene expression compared with major allele C. They also found that there was a difference in allele frequency between hypertensive and normotensive subjects in an Australian population. In hypertensive subjects the frequency of the T allele was 13.3%, compared with 7.6% in the normotensive subjects ( $P < 0.001$ ) [10,17].

The frequencies of the SLC7A1 gene variants in our study population were similar to those reported previously [10]. In contrast to the earlier study [10], polymorphism rs41318021 in the SLC7A1 was not associated with essential hypertension in 50-year-old subjects. On the other hand, a borderline effect of this variation was seen in these same subjects from the age of 35 years. This is in line with the conclusion that the effect of a single gene polymorphism is seen more likely in a younger population [5]. The observed effect of a single gene variation on blood pressure in GWAS studies is low: +1.16 mmHg at the highest [5], as also seen in the present study.

The background of hypertension consists of many genes and moreover of gene-environment interactions. Ageing is known to result in untoward increases in body mass index in the TAMRISK study cohorts [11]. Thus, genetic effects are more likely to be seen at younger ages. With aging, other factors, e.g. overweight, may be somewhat more relevant to development of hypertension.

## Conclusions

In conclusion, the effect of gene variation rs41318021 in the human SLC7A1 on blood pressure was of borderline significance at the age of 35 years, and masked by

environmental factors by the age of 50 years in the TAMRISK study.

## Consent

Written informed consent was obtained from all study subjects for the publication of this report.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

KM contributed to the analysis and interpretation of the data and drafting the manuscript. TK and STN contributed to conception and design of this study, drafting the manuscript and revising the article critically for important intellectual content. All authors read and approved the final manuscript.

## Authors' information

Tarja Kunnas and Seppo T Nikkari are senior authors.

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# Genetic Variant Coding for Iron Regulatory Protein HFE Contributes to Hypertension, the TAMRISK Study

Kirsi M. Määtä, MB, Seppo T. Nikkari, MD, PhD, and Tarja A. Kunnas, PhD

**Abstract:** Iron is essential for body homeostasis, but iron overload may lead to metabolic abnormalities and thus increase the risk for atherosclerosis and many other diseases. Major histocompatibility complex class I-like transmembrane protein (HFE) is involved in body iron metabolism. The gene coding for HFE has 3 well-known polymorphic sites of which H63D (rs1799945, C > G) has recently been associated with hypertension in a genome-wide association study (GWAS) study. In the present study, we wanted to clarify whether the genetic variant associates with hypertension in a Finnish cohort consisting of 50-year-old men and women.

The study included 399 hypertensive cases and 751 controls from the Tampere adult population cardiovascular risk study (TAMRISK) cohort. Genotyping of polymorphisms was done by polymerase chain reaction using DNAs extracted from buccal swabs.

We found that individuals with the mutated form of the H63D polymorphic site (G-allele) had a 1.4-fold risk ( $P=0.037$ , 95% confidence interval [CI] 1.02–1.89) for hypertension at the age of 50 years compared with the CC genotype carriers. When obese subjects (body mass index > 30 kg/m<sup>2</sup>) were analyzed in their own group, the risk for hypertension was even stronger (odds ratio 4.15,  $P<0.001$ , 95% CI 1.98–8.68). We also noticed that the blood pressure (BP) readings were higher in those with the minor G-allele when compared to ones having a normal genotype already at the age of 35 years. Means of systolic/diastolic BPs were 127/81 mm Hg for CC and 131/83 mm Hg for CG + GG groups ( $P<0.001$  for systolic and  $P=0.005$  for diastolic pressure).

In conclusion, HFE genetic variant H63D was associated with essential hypertension in Finnish subjects from the TAMRISK cohort confirming a previous GWAS study. The effect of this SNP on BP was also confirmed from a longitudinal study.

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**Abbreviations:** BMI = body mass index, CI = confidence interval, HFE = histocompatibility complex class I-like transmembrane protein, PHE = periodic health examination.

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The authors have no conflicts of interest to disclose.

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## INTRODUCTION

Hypertension has a substantial impact on public health because of its high prevalence and associated complications. Efforts for the discovery of genes affecting hypertension have been conducted, but the vast majority of genetic contribution to blood pressure (BP) remains still unexplained.<sup>1</sup> The major lifestyle contributors to hypertension in Western countries are overweight, physical inactivity, high salt intake, and low potassium intake.<sup>2</sup> However, less attention has been allocated to iron metabolism and especially of the effects of mild iron overload on hypertension.

Iron is essential for mammalian homeostasis because of its presence in hemoglobin for oxygen transport, and in many biochemical reactions. Iron uptake occurs in duodenum and jejunum and it is mediated by transport proteins, such as ferroportin.<sup>3,4</sup> Iron is stored in hepatocytes and macrophages within polymers of ferritin. Excess iron in the body leads to iron accumulation in the liver and other organs and may cause, among others, diabetes, liver cirrhosis, bone and joint disease, heart disease, and hepatocellular carcinoma.<sup>5,6</sup>

HFE is a major histocompatibility complex class I-like transmembrane protein, which associates with class I light-chain  $\beta$ 2-microglobulin.<sup>7</sup> HFE mediates cell uptake of transferrin-bound iron. HFE also modulates expression of the key regulator of plasma iron, hepcidin.<sup>6,8,9</sup> Two most common mutations in the HFE gene are C282Y (rs1800562) and H63D (rs1799945), of which the C282Y has been found as the major mutation behind significant iron overload. However, also the H63D mutation leads to dysfunctional HFE protein,<sup>10</sup> which could result in mild iron overload. The carrier frequency of the H63D mutation is 8.1% worldwide and 14% to 22% in European populations.<sup>11,12</sup> There are only a few previous studies that have addressed the association of HFE-mediated dysfunctional iron regulation with hypertension. Ellervik et al<sup>13</sup> showed that men homozygous for the C282Y allele had increased prevalence of antihypertensive medication >55 years of age. A recent genome-wide association study (GWAS) has given evidence that H63D mutation in the HFE gene is associated with BP.<sup>14</sup>

In the present study, we wanted to study a possible association between H63D polymorphism and hypertension in the Finnish Tampere adult population cardiovascular risk study (TAMRISK) cohort.

## METHODS

### Participants

The TAMRISK is a prospective, longitudinal population-based health survey study in Tampere, a city in southern Finland with a population of 210,000. The Tampere city health care center has provided regular periodic health examinations (PHEs) for screening and counseling for the adult population of the city since 1980.

The PHE included one 60-minute session with a public health nurse at the center's health examination unit. A standard questionnaire on subjective health and health behavior and referral to basic laboratory tests were sent with the invitation letter. In the session, the questionnaire information and screening tests were reviewed. Counseling was given on topics selected by the client and also on findings of the screening tests. Information on current and previous diseases was based on self-report of diagnosis by a physician, including history of coronary artery disease, myocardial infarction, and diabetes. Family history of hypertension in a close relative was also asked in the questionnaire. The frequency of physical exercise comprised both leisure and commute-related activity. Physical examination included a single BP measurement (mm Hg) using a calibrated mercury sphygmomanometer. Height (cm) and weight (kg) were recorded from which the body mass index (BMI) was calculated. Informed consent was obtained at the time of the physical examination for use of the data for research.

The data for the TAMRISK study was collected from the PHEs done for 50-year-old men and women living in Tampere. TAMRISK data includes information of risk factors for hypertension: BP, weight, family history of cardiovascular diseases, lipid values and smoking, diabetes, and exercise habits.

Buccal swabs for DNA extraction and a permission form to use PHE data were collected by mail separately of the physical examination. The DNA samples were collected during 2006 and 2010. Informed consent was obtained from all participants. The Ethics Committees of the Tampere University Hospital and the City of Tampere approved the study.

Cases ( $n = 399$ ) in this study were the subjects who had hypertension at the age of 50 (as diagnosed by a physician) and for each case, at least 1 normotensive control ( $n = 751$ ) with the same sex and similar smoking habits were chosen from a PHE cohort ( $n = 6000$ ). Smoking status was evaluated based on self-reporting. Finally, we selected a subpopulation of men and women who had available PHE data at the ages of 35, 40, 45, and 50 years.

## Genotyping

DNA was extracted from buccal swabs using a commercial kit (Qiagen Inc, Valencia, CA). Genotyping was performed using primers 2A (5'-ACA TGG TTA AGG CCT GTT GC-3') and 2B (5'-GCC ACATCT GGC TTG AAA TT-3'). Amplification was done in 94°C for 5 minutes followed by 36 cycles of 94°C, 61°C, and 72°C for 30 seconds each. The final extension was at 72°C for 7 minutes. The polymerase chain reaction products were digested with BclI. Digested products were run in 2% agarose gel electrophoresis.

## Statistical Analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Inc, Chicago, IL). Clinical characteristics of cases and controls at the age of 50 years were compared by analysis of variance (ANOVA). The comparison of categorical variables and deviation from Hardy-Weinberg equilibrium was performed using  $\chi^2$  statistics. The association of HFE gene variants with hypertension risk was analyzed using binary logistic (forward conditional) regression analysis. The model included H63D variation, BMI, and family history of hypertension as explainable variables. The ANOVA for repeated measures was used to assess the differences in mean BPs between genotypes at the age of 35, 40, 45, and 50 years. The model included the main effects of group factor and time, and their interaction. *P* values were considered significant when  $\leq 0.05$ .

## RESULTS

Clinical characteristics of the 399 cases and 751 controls are presented in Table 1. In the whole study population, frequencies of the H63D variants were 0.74 for genotype CC ( $n = 851$ ), 0.24 for CG ( $n = 280$ ), and 0.017 for GG ( $n = 19$ ). The distributions of the genotypes followed the Hardy-Weinberg equilibrium ( $P = 0.55$ ). When genotype frequencies of the cases were compared with controls, they significantly differed between the study groups ( $P = 0.04$ ). In the hypertension group, frequencies were 0.70 for CC ( $n = 279$ ), 0.28 for CG ( $n = 112$ ),

**TABLE 1.** Clinical Characteristics of Cases and Controls of the Study Population

Clinical Characteristic	Cases (n = 399)	Controls (n = 751)	<i>P</i> Value
Age, y	50.0 ± 0	50.0 ± 0	
Body mass index, kg/m <sup>2</sup>	28.5 ± 5.1	25.4 ± 3.6	<0.001
Cholesterol, mmol/L	5.4 ± 1.0	5.4 ± 0.9	0.9
HDL cholesterol, mmol/L	1.6 ± 0.4	1.7 ± 0.4	<0.001
LDL cholesterol, mmol/L	3.2 ± 0.9	3.2 ± 0.8	0.6
Triglycerides, mmol/L	1.5 ± 1.2	1.2 ± 0.7	<0.001
Glucose, mmol/L	5.2 ± 1.2	4.8 ± 0.6	<0.001
Systolic blood pressure, mm Hg	143.0 ± 16.2	129.8 ± 14.7	<0.001
Diastolic blood pressure, mm Hg	93.0 ± 8.9	84.4 ± 9.4	<0.001
Hypertension (%)	100	0	<0.001
Daily smokers (%)	26.1	23.2	0.3
Exercise (at least twice a week) (%)	66.2	71.9	0.06
Diabetes (%)	7.1	0.0	<0.001
Lipid-lowering drugs (%)	15.1	3.5	<0.001
HFE allele distribution, rs1799945: C/G	0.84/0.16	0.87/0.13	0.025

HDL = high-density lipoprotein, HFE = histocompatibility complex class I-like transmembrane protein, LDL = low-density lipoprotein. Data is presented as mean ± standard deviation.

**TABLE 2.** Adjusted OR Results Obtained from Logistic Regression Models (Forward Conditional) for Hypertension

Model	n	Factor	OR	95% CI	P Value
Model 1; (all participants)	1150	BMI	1.19	1.15–1.23	<0.001
		Family history	3.60	2.71–4.78	<0.001
		rs1799945 (G-allele)	1.39	1.02–1.89	0.037
Model 2; BMI > 25 kg/m <sup>2</sup>	663	BMI	1.23	1.16–1.29	<0.001
		Family history	3.66	2.58–5.22	<0.001
		rs1799945 (G-allele)	1.61	1.10–2.34	0.013
Model 3; BMI > 30 kg/m <sup>2</sup>	269	BMI	1.17	1.06–1.29	0.002
		Family history	2.91	1.54–5.51	0.001
		rs1799945 (G-allele)	4.15	1.98–8.68	<0.001

BMI = body mass index, CI = confidence interval, OR = odds ratio.

and 0.02 for GG (n = 8), whereas in the control group they were 0.76 for CC (n = 572), 0.22 for CG (n = 168), and 0.015 for GG (n = 11). Because of low number of GG gene variants, CG and GG were combined for further analysis.

To find the interpretative factors for hypertension, we used logistic regression analysis. When the association of H63D was defined alone, the risk for hypertension among G-allele carriers was 1.4-fold ( $P = 0.018$ , 95% confidence interval 1.06–1.82) compared with CC genotype carriers. When H63D variants, family history of hypertension, and BMI were included into logistic regression analysis as factors (forward conditional), adjusted odds ratios (ORs) for hypertension were 1.4 for H63D G-allele carriers, 3.6 for family history of hypertension, and 1.2 for BMI (model 1, Table 2).

The association of HFE gene variants with hypertension was further calculated separately for the participants who were overweight or obese (model 2; BMI > 25, n = 663), and finally only for those who were obese (model 3; BMI > 30, n = 269). In these subgroup analyses, the risk for hypertension at the age of 50 years was even stronger among G-allele carriers (Table 2).

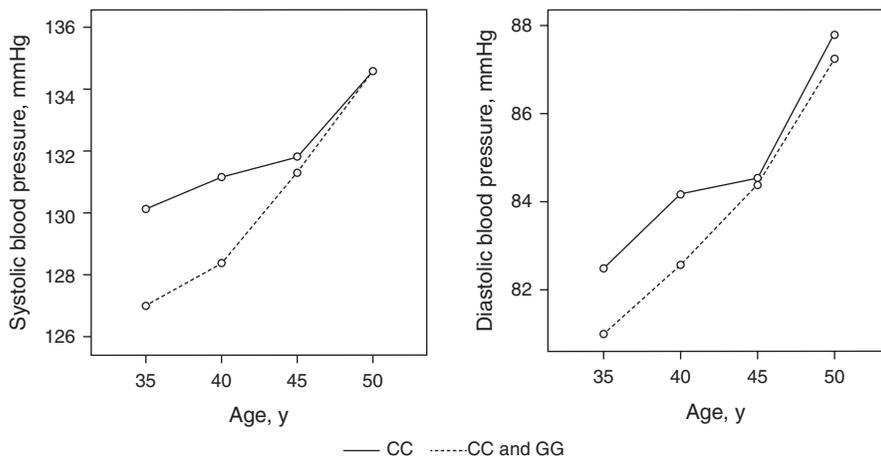
Next we analyzed BP data from the ages of 35, 40, 45, and 50 years. Systolic and diastolic BPs at the age of 35 years were found to be significantly different between the 2 genetic variant groups. Means of systolic and diastolic pressures were 127/81 mm Hg for the CC group and 131/83 mm Hg for the CG + GG group ( $P < 0.001$  for systolic and  $P = 0.005$  for diastolic pressure).

The difference remained significant also at the age of 40 years for systolic BP ( $P = 0.027$ ), but disappeared at the ages of 45 and 50 years (Figure 1). By analysis of repeated measures, there was no statistically significant interaction in the change of systolic/diastolic BP between the variants and the follow-up time (Figure 1).

### DISCUSSION

The present study suggests that mutation in the HFE gene is associated significantly with hypertension among a 50-year-old Finnish population. This finding is in line with a recent GWAS study.<sup>14</sup> In addition, we also noticed that subjects with the mutated variant of the gene had higher BPs already at the age of 35 years. Previous studies of the HFE gene have concentrated mainly on hemochromatosis, but we wanted to have a different point of view into the effects of possible mild iron overload on the risk of hypertension.

Genes behind hypertension may cause significantly higher BP already at young age and lead to severe consequences. Hypertension is a significant risk factor for heart disease, stroke, and renal disease.<sup>15</sup> In our study, both systolic and diastolic BPs were significantly higher already at the age of 35 years in the mutated HFE gene group (CG + GG), the difference being 4/2 mm Hg respectively as compared to normal gene carriers. At the ages of 45 and 50 years, no significant difference was found. A possible difference in BP after 45 might be difficult to establish,



**FIGURE 1.** Means of systolic and diastolic blood pressures at different ages according to HFE (H63D) genetic variants.

since many of the subjects who had hypertension were already on BP medication. It has been shown that a prolonged increase even as small as 2 mm Hg in systolic BP is involved with 10% higher stroke mortality and 7% higher mortality for other vascular causes such as ischemic heart disease in middle age.<sup>16</sup>

It is known that obesity is a major risk factor to develop hypertension, which was also seen in our study. However, the effect of HFE H63D variant on hypertension was statistically significant even when BMI was taken into account in the analyses. In addition, we found that when overweight and obese subjects were analyzed in their own group, the effect of G-allele in predicting hypertension was even stronger compared with CC-genotype. The highest risk for developing hypertension was obtained among obese subjects (OR 4.2 for carriers of the H63D G-allele), which was greater than the corresponding OR of BMI. Although it is known that ferritin levels are enhanced in obese subjects,<sup>17</sup> a mechanism by which obesity could modulate the effect of the HFE polymorphism is not known. However, an obesity-associated effect on hypertension has been shown for SELE 554L/F<sup>18</sup> and TMEM182,<sup>19</sup> which are genes integrating genetic factors and obesity in the development of hypertension.

One possible explanation for higher BP of carriers of the H63D mutation is mild iron overload. Ferrous iron is absorbed into enterocytes in the duodenum and exported into circulation via ferroportin. In the circulation, iron is bound to transferrin.<sup>8</sup> The mechanisms of how different H63D variants participate in the control of iron metabolism have not been fully explained. It is assumed that regulation occurs mainly via hepcidin, which is an iron-regulating hormone.<sup>5</sup> Hepcidin levels increase due to rising body iron burden and mutations in the *HFE* gene block hepcidin acting as a feedback inhibitor of iron absorption. Therefore, HFE deficiency may lead to iron accumulation.<sup>3,5,20</sup>

The impact of H63D mutation on iron overload has been studied with controversial results.<sup>21–23</sup> According to a large meta-analysis, homozygosity of the mutated form of H63D was associated with iron overload, but heterozygosity conferred no risk.<sup>24</sup> Gochee et al<sup>21</sup> suggested that H63D mutation is insufficient in itself to cause significant iron overload and that additional modifying factors are needed. Aguilar-Martinez et al<sup>25</sup> searched for genetic modifiers affecting H63D mutation homozygotes, but found no link to potential genetic modifiers within the HFE or other genes. Blood tests for assessing body iron levels reflecting iron overload include serum ferritin and transferrin saturation.<sup>24</sup> A limitation of the TAMRISK study population is the lack of these measurements of saturation. However, it has previously been published that men with essential hypertension had greater iron stores than normotensive controls.<sup>26</sup> In addition, Cash et al<sup>27</sup> observed that BPs were elevated in hemochromatosis patients when compared with controls, mean difference being 10 mm Hg for systolic and 7.9 mm Hg for diastolic BPs. This could suggest a relationship between iron overload and hypertension.

Excess iron in plasma produces reactive oxygen species, which are a possible explanation for organ damage in iron overload.<sup>28</sup> Oxidative stress also induces cardiovascular and renal injury, and activates sympathetic nervous system leading to increase in BP. No sole evidence of oxidative stress in the pathogenesis of hypertension exists in humans, but animal studies suggest a causative role.<sup>29</sup> In the study by Liu et al<sup>30</sup>, mutated H63D protein was associated with prolonged endoplasmic stress and elevated iron levels in a transgenic mouse model.

Although the mechanism is not yet known, our results suggests that the minor G-allele of the H63D variant

(rs1799945) is associated with higher risk for hypertension at the age of 50 years, compared with the CC-genotype carriers. In addition, we found that individuals with the G-allele had also higher BP already at the age of 35 years. Although the difference in systolic and diastolic BP between different genotype carriers was small, the long-term consequences may be remarkable. Further studies should include measurements of plasmatic iron to prove without doubt the relationship between the presence of the allele, mild iron overload, and hypertension. If this mechanism is through iron overload, subjects with mutated H63D should possibly restrict their supply of iron.

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RESEARCH ARTICLE

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# Periodic cohort health examinations in the TAMRISK study show untoward increases in body mass index and blood pressure during 15 years of follow-up

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## Abstract

**Background:** Obesity is a significant risk factor for hypertension and diabetes. A cohort of 50-year-old voluntary periodic health examination (PHE) participants was analyzed 15 years retrospectively. Our aim was to evaluate changes in body mass index (BMI) and blood pressure in subjects diagnosed with hypertension and/or diabetes in comparison with healthy controls.

**Methods:** Voluntary periodic health examinations (PHE) of the citizens have been carried out by the city of Tampere, Finland. Health data, including body mass index (BMI) and blood pressure, were recorded every five years, starting at the age of 35 (baseline). A total of 339 subjects from the 50-year-old cohort having hypertension and/or diabetes were chosen to the study group. The control group included 604 subjects from the 50-year-old cohort who had the same follow-up information but were not diagnosed with hypertension and/or diabetes.

**Results:** In the study group the mean BMI had increased from 26.1 at baseline to 28.5 at the final 15-year follow-up examination. The corresponding increase in the control group was from 23.8 at baseline to 25.5 at the final follow-up. The difference in change with time between the groups was statistically significant ( $p = 0.04$ ). On the average, the controls gained 4.9 kilograms, whereas subjects in the study group gained 7.0 kilograms over the 15 years of follow-up. Systolic and diastolic blood pressures were also higher in the study group already at baseline and systolic blood pressure increased with time more in the study group than in the control group ( $p = 0.004$ ).

**Conclusions:** BMI and blood pressure were higher in the study group in comparison with the controls already at baseline at 35 years, and the differences were not favorably changed during the follow-up. Apparently, the effect of PHE had not been as efficient as planned on subjects in the study group, who were already slightly overweight at baseline.

**Keywords:** Periodic health examinations, Body mass index, Hypertension

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## Background

Periodic health examinations (PHE) are endorsed as a means of preventive medicine and usually include medical history, physical examination and multiple screening using simple laboratory tests. Goals of PHE are early diagnosis of chronic diseases and the improving of patient understanding of health and disease [1].

PHE has been a fundamental part of medical practice for decades despite a lack of consensus on its value. In Canada, the roots of the annual PHE date back to 1861. In the 1970s and 1980s, both the Canadian Task Force on the Periodic Health Examination and the United States Preventive Services Task Force recommended abandoning the PHE in favor of regular appropriate evidence-based preventive care during regular visits [2,3]. A recent systematic review of medical databases suggests that the PHE may lessen patient worry, but other outcomes were vague [4]. It is clear that additional research is needed to assess the costs, benefits and harms as well as long-term outcomes, of the PHE [5].

Obesity is a significant risk factor for chronic diseases, such as hypertension and diabetes [6-8]. More than 50% of European adults are obese or overweight [9]. We evaluated changes in body mass index (BMI) and blood pressure during 15 years of PHE follow-up. Subjects diagnosed with hypertension and/or diabetes by the age of 50 were compared with controls.

## Methods

The Tampere adult population cardiovascular risk study (TAMRISK) is a prospective, longitudinal population based health survey study in Tampere, a city in southern Finland with a population of 210 000 [10,11].

The Tampere city health care centre has provided regular PHEs, for screening and counselling, for the adult population of the city since 1980. All 40- and 50-year-old inhabitants have been invited to participate in these health surveys. During some periods, also 35- and 45-year-olds have been invited to participate. The PHE consisted of one 60-minute session with a public health nurse at the centre's health examination unit. In the session, the questionnaire information and screening tests were reviewed. Counseling was given on topics selected by the participant and also on findings of the screening tests.

Information is available for some participants for 15 years of consecutive 5-year follow-ups concerning risk factors for cardiovascular diseases, including family history, blood pressure, lipid values, smoking, waist circumference, diabetes exercise and eating habits. Also data on diseases has been recorded. Presently, invitations for the survey are sent to all subjects from Tampere that are 40 or 50 years old that year.

All subjects who had a self-reported diagnosis of hypertension and/or diabetes (made by a physician,  $n = 681$ ) were chosen from a PHE 50-year-old cohort ( $n = 6000$ ). Those subjects who also had information on 35-, 40-, and 45- year PHE formed the study group ( $n = 339$ ). The control group included subjects from the same 50-year-old cohort who were apparently healthy, and had the same follow-up information. Controls were chosen for every subject in the study group by matching them according to sex ( $n = 604$ ). Baseline clinical examinations had taken place during the calendar year when the subject caught the age of 35 in 1988–91. At the age of 40 the same subjects were invited to health examination again if they still lived in Tampere. This happened in 1993–96. The third follow up took place in 1998–2001 at the age of 45. The last follow up was in years 2003–06 at the age of 50. The follow-up time was 15 years. The Ethics Committees of the Tampere University Hospital and the City of Tampere approved the study. Written informed consent for participation in the study was obtained from all participants.

## Baseline measurements

The basic evaluation in 1988–91 included an interview by a public health nurse. The interview was conducted using a structured questionnaire about health and health-related behavior, including questions about current and previous diseases. Information on current and previous diseases was based on self-report of diagnosis by a physician, including history of MI and diabetes. The questionnaire also assessed symptoms and ailments experienced within the past six months. These included questions of health in general and mental health. Questions of health-related behavior included current and past smoking. The frequency of physical exercise comprised both leisure and commute related activity. Quantitative estimation of the alcohol intake was carried out by using three structured questions to determine the amount and frequency of drinking. The total mean consumption of all alcoholic drinks was used, expressed as grams of pure ethanol per week. Physical examination included a single blood pressure (BP) measurement (mm of mercury) using a calibrated mercury sphygmomanometer. Serum total cholesterol (mmoles/liter) was measured by enzymatic techniques. Height (cm) and weight (kg) were recorded from which the body mass index was calculated.

## Statistical analyses

The description of data was made by means and standard deviations for continuous variables and by proportions for categorical variables. The descriptions were constructed separately to the study group and controls. The differences in dichotomous variables were compared with

Fisher's exact test, in other categorical variables with Chi-square test and in continuous variables with *t*-test, or Mann Whitney *U* test if the distribution was skewed. The analysis of variance for repeated measures was used to assess the differences in changes of body mass index and blood pressure between the study group and the control group during the follow up time. Normality of the distributions of the dependent variables were tested using Kolmogorov-Smirnov test, and logarithmic transformation was applied if the distribution of the variable was skewed. The changes were calculated in relation to the baseline measurement at the age of 40, 45 and 50. The model included the main effects of group factor and time, and their interaction. The baseline measurement of dependent variable was included in the model as a covariate. The risk level in the analyses was set equal to *p*-value of 0.05. Analyses were made using SPSS for Windows (version 16.0) program.

## Results

The characteristics of the 339 cases belonging to the now 50-year-old study group with diagnosed hypertension (91%), diabetes (15%) or both (9%), and their 604 controls are shown in Table 1 at baseline, when they were 35 years old. At the age of 35, subjects in the study group had already higher mean BMI, systolic and

**Table 1 Background characteristics of subjects examined at baseline in 1988–91 at the age of 35 stratified by whether or not they had hypertension or diabetes (as diagnosed by a physician) by the age of 50**

	Study group	Controls	<i>p</i> *
N	339	604	
Male gender (%)	62	61	0.726
Married (%)	82	83	0.647
In working life (%)	96	93	0.207
Physical exercise at least 3 times/week (%)	25	28	0.442
Current daily smokers (%)	33	28	0.226
Self-reported health status at least quite good (%)	69	80	0.004
Alcohol consumption as grams/week (SD)	95.3 (96.2)	79.2 (90.0)	0.047
Height (cm), mean (SD)	172.8 (9.5)	172.8 (9.2)	0.910
BMI (kg/m <sup>2</sup> ), mean (SD)	26.1 (4.4)	23.8 (3.2)	<0.001
Diastolic BP (mm Hg), mean (SD)	86 (10)	78 (8)	<0.001
Systolic BP (mm Hg), mean (SD)	135 (13)	124 (10)	<0.001
Hypertension (%)	9	0	<0.001
Diabetes (%)	2	0	0.016
Serum cholesterol (mmol/l), mean (SD)	5.5 (1.0)	5.2 (1.0)	0.001

\*Fisher's test for categorized variables, Mann Whitney *U* test for continuous variables. BP, blood pressure; BMI, body mass index.

diastolic blood pressure and serum cholesterol as compared to controls. Physical exercise and daily smoking did not differ between the groups at the age of 35 or during the whole follow-up. In the study group the self-reported health status was poorer and more alcohol was used per week compared to controls at the age of 35. This difference persisted also at the age of 50 (data not shown). In the study group there were already 9 subjects with diagnosed hypertension and two with diabetes at the age of 35. Subsequently, the mean BMI increased from 26.1 at baseline to 28.5 at the last follow-up examination in the study group, whereas in the 604 controls the corresponding increase was from 23.8 at baseline to 25.5 at the last follow-up. On average, controls gained 4.9 kilograms, whereas subjects with hypertension or diabetes at the age of 50 gained 7.0 kilograms over the 15 years of follow-up.

The mean changes in BMI and blood pressure are shown in Table 2. We found a statistically significant interaction in the change of BMI between the study group and the control group, and the follow-up time (*p* = 0.04). The BMI increased more in the study group than in the control group, and the increase rate was constant over the follow-up time. In the control group BMI also increased, but the increase rate was lower especially during the last 5 years of the follow-up (Figure 1).

A statistically significant interaction was found in the change of systolic blood pressure between the study group and the controls, and the follow-up time (*p* = 0.004). The increase was slightly higher in the study group than in the control group. In the study group the highest increase was found between 40 and 45 years of age, whereas in the control group the increase rate was at the highest between the age of 45 and 50.

The diastolic blood pressure remained higher in the study group than in the control group during the follow-up time, but the interaction in the change between the group and the time effects was not statistically significant (*p* = 0.06). At the age of 50, a total of 61% of the study group were on self-reported blood pressure medication.

## Discussion

A difference was found in the change of BMI between the study group and the control group during the follow-up time. In the study group the increase rate was higher and it was constant over the follow-up time, whereas in the control group the rate slowed slightly during the last 5 years of the follow-up. This suggests that the effects of the PHE had not been efficient enough to stop the weight gain in either group. Especially in the study group the remarkable increase in BMI was seen despite the PHE conducted during the follow-up time and there were no signals of weight gain slowing down.

**Table 2 The change in body mass index (BMI) and blood pressure in subjects from the TAMRISK study stratified by whether or not they had hypertension or diabetes by the age of 50**

	Study group	Controls	P-value (interaction in change between group and time effects)	Difference (95% CI)	P-value (t-test)
Change in BMI (kg/m <sup>2</sup> )			0.04		
40 years of age	0.9 (2.3)	0.4 (1.8)		0.43 (0.14;0.72)	0.008
45 years of age	1.8 (2.4)	1.3 (1.8)		0.59 (0.30;0.87)	<0.001
50 years of age	2.4 (3.1)	1.7 (2.2)		0.73 (0.36;1.11)	<0.001
Change in systolic blood pressure (mmHg)			0.004		
40 years of age	2.5 (10.5)	0.6 (8.2)		1.95 (0.54;3.36)	0.007
45 years of age	6.2 (15.5)	2.2 (10.7)		3.99 (1.96;6.03)	<0.001
50 years of age	6.2 (20.4)	5.3 (14.1)		0.90 (-1.73;3.53)	0.501
Change in diastolic blood pressure (mmHg)			0.06		
40 years of age	2.6 (10.0)	1.4 (7.1)		1.17 (-0.14;2.48)	0.080
45 years of age	4.5 (11.6)	2.4 (7.4)		2.08 (0.58;3.57)	0.007
50 years of age	5.6 (14.0)	5.7 (9.2)		-0.16 (-1.95;1.63)	0.859

The change was calculated in relation to baseline (at 35 years of age) during the 15-year follow-up when the periodic health examinations were conducted. Positive values reflect the increase in BMI or blood pressure. Values are means (standard deviations).

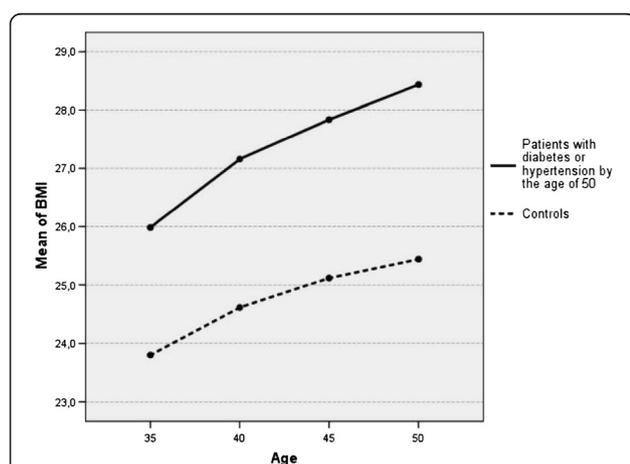
The subjects in the study group had a higher level of BMI at baseline. Because obesity is known to be a major risk factor for cardiovascular disease and diabetes, the subjects in the study group were in greater risk already on that basis compared with the control group at the very beginning. In that sense it would have been possible to allocate the preventive actions to these subjects in the PHEs. The result suggests that PHE could not intervene with the weight gain of subjects who had a high risk to cardiovascular disease and diabetes.

The increase in blood pressure was in high extent consistent with that of BMI. The blood pressure (both systolic and diastolic) was at higher level in the study group than in the control group already at baseline. The

increase in systolic blood pressure was also greater in the study group than in the control group between the ages of 40 and 45. Favorable effects of PHE were not seen in relation to changes in blood pressure, even though blood pressure medication had been started for more than half of the subjects in the study group.

Participant views of the PHE in Tampere have been previously examined in 1995, in a 45-year-old cohort [12]. According to the participants' accounts, the discussions and counseling with a public health nurse had dealt with living habits and, to a lesser extent, with diseases, symptoms and the prevention of illness. Virtually all respondents saw the PHE program as beneficial for everyone. Opinions were divided on its benefits compared with medical help from a physician. Looking at the present results, the PHE, conducted at 5-year intervals by a public health nurse, had not been very effective in intervening with living habits, since BMI continued to increase in the study group, who were already at risk at the age of 35. It has been reported that adults who were overweight but not obese (i.e.,  $25.0 \leq \text{BMI} \leq 29.9$ ) were at significantly increased risk of developing numerous health conditions, such as diabetes, gallstones, hypertension, heart disease, and stroke [6]. Although the study group had a diagnosis of diabetes or hypertension by a physician, their medical intervention was not sufficient in terms of blood pressure medication. Certainly a larger proportion of cases would have been in need for more regular intervention and medication.

At the age of 35, subjects in the study group used more alcohol per week (95 g) than controls (79 g). This alcohol consumption should be considered in the context that self-reporting of drinking is commonly



**Figure 1** The development of body mass index (BMI) in the study group and controls during the time the periodic health examinations were conducted.

unreliable and the official per capita mean consumption of absolute ethanol in Finland for inhabitants 15 years and older is 8.4 l per year, corresponding to 162 g per week [13]. Since physical activity was not different between the study group and controls, the difference in alcohol intake has potential to explain the remarkable increase of BMI in the study group compared to controls during the follow-up time.

The strength of present study is the large cohort from which the data was collected. The data analyzed included 943 subjects all born in the four year period 1951–54 and followed up for 15 years. Thus there was no confounding by age. The 15-year follow-up of the cohort enables to study trends in health factors. However, since the study group was restricted to residents of a large city in Finland poses a challenge to how broadly one can apply the findings. Because the studied population represented only Caucasians, our findings cannot be generalized to minority groups.

## Conclusions

The unfavorable differences in BMI and blood pressure between the study group, who had hypertension and/or diabetes at the age of 50, and the controls were seen already at the 35-year-old PHE and they were not corrected during the 15-year follow-up time when PHE were conducted. This suggests that the effect of PHE had not been as favorable as desired among the subjects in the study group, who were at risk already at the age of 35.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

TK, KM, PP and SN had substantial contributions to conception and design and interpretation of data and writing the manuscript. All authors read and approved the final manuscript.

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