

ELINA HAUTANIEMI

Haemodynamic Influences of Liquorice and Fermented Milk Products Containing Lactotripeptides and Plant Sterol Esters

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Fermented Milk Products
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and Plant Sterol Esters

ACADEMIC DISSERTATION

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ABSTRACT

Hypertension is a significant predisposing factor for cardiovascular disease, which is a leading cause for death worldwide. Elevation of blood pressure (BP) often coincides with abdominal obesity, insulin resistance, and dyslipidaemia known as the metabolic syndrome (MetS), which is associated with increased risk of cardiovascular disease and type 2 diabetes. Lifestyle modifications including a healthy balanced dietary pattern is central for both prevention and treatment of hypertension and related risk factors. As possible functional foods with antihypertensive properties, milk casein-derived lactotripeptides (LTPs) isoleucine-proline-proline and valine-proline-proline have been suggested for the dietary treatment of prehypertension and mild hypertension together with other lifestyle changes. The proposed mechanism of action includes inhibition of the angiotensin-converting enzyme. However, the BP lowering efficacy of LTPs has not been consistent in all studies.

Liquorice ingestion has been recognised as a dietary-based reason for elevation of BP. The active metabolite of liquorice products, glycyrrhetic acid, inhibits the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2), which degrades active cortisol to inactive cortisone. Thus, liquorice ingestion results in enhanced activation of the mineralocorticoid receptor (MR) by cortisol, although the physiologic agonist of the MR is aldosterone. The MRs are co-expressed with the 11 β -HSD2 in tissues involved in the regulation of BP including the kidneys, vascular wall, central nervous system, and the heart. Thus, the elevation of BP following liquorice ingestion could be mediated via sodium and water retention, increased peripheral arterial resistance and large arterial stiffness, or changes in the autonomic tone and cardiac function.

The aim of the present thesis was to study the influences of simultaneous intake of LTPs that have been previously suggested to inhibit the renin-angiotensin-aldosterone system (RAAS), and plant sterol esters (PSe) that are known to decrease the absorption of dietary and endogenous cholesterol, on haemodynamics and plasma lipids in subjects with the MetS. In addition, the detailed haemodynamic changes underlying the liquorice-induced elevation of BP, which is characterised by the suppression of the RAAS, were investigated in healthy volunteers.

A study population of 104 subjects with the MetS were randomised to three parallel groups to consume fermented milk product containing either 5 or 25 mg/d of LTPs combined with 2 g/d of PSe, or placebo for 12 weeks, in a double-blind fashion (**I**). In addition, 22 normotensive subjects consumed 120-300 g/d of liquorice (corresponding to 290-370 mg/d glycyrrhizin) for two weeks in parallel with a control group maintaining their habitual diet (**II-IV**). Haemodynamics were measured non-invasively at rest (**II**) and during orthostatic challenge (**I, III, IV**) in the laboratory, and brachial BP at home (**I**). In study IV, the haemodynamic recordings were performed in the absence and presence of sublingual nitroglycerin and inhaled salbutamol to investigate the possible alterations in the vasodilatory responses induced by the aforementioned drugs after liquorice intake. Also, the urinary ratio of cortisone to cortisol metabolites, a marker of the inhibition of the enzyme 11 β -HSD2, was examined in relation to the haemodynamic findings (**IV**).

In study I, the test products induced no significant effect on home BP measurements, or on BP, cardiac index, and systemic vascular resistance index (SVRI) in the laboratory during the haemodynamic recording protocol compared to placebo. However, a slight reduction in plasma LDL-cholesterol was observed following intake of fermented milk products containing LTPs and PSe.

In study II, liquorice ingestion was found to increase extracellular water volume, elevate central pulse pressure and augmentation index indicating enhanced central wave reflection, and elevate peripheral and central BP. The findings of study III indicated that liquorice exposure also elevated SVRI and increased large arterial stiffness. In addition, enhanced augmentation index and reduced heart rate was especially observed in the upright position (**III**). The liquorice-induced impairment in cardiac chronotropic response to upright posture was found to correlate with the decrease in urinary ratio of cortisone to cortisol metabolites (**IV**). Also, the liquorice-induced increase in SVRI was detected in the absence and presence of nitroglycerin, while in the presence of salbutamol there were no differences between the level of SVRI measured before and after liquorice exposure (**IV**).

In conclusion, in subjects with the MetS, intake of fermented milk products containing LTPs and PSe showed no antihypertensive effect, while a mild LDL-cholesterol lowering effect was detected. Two weeks of daily liquorice intake elevated BP through multiple mechanisms including increased extracellular water volume, amplified pressure wave reflection from the periphery especially in the upright position, increased large arterial stiffness, and elevated peripheral arterial resistance via impaired response to nitric oxide. The measurement of mere supine

haemodynamics would significantly underestimate the cardiovascular influences of liquorice intake.

TIIVISTELMÄ

Kohonnut verenpaine on merkittävä riskitekijä sydän- ja verisuonisairauksille sekä ennenaikaiselle kuolemalle maailmanlaajuisesti. Kohonnut verenpaine esiintyy usein yhdessä keskivartalolihavuuden, dyslipidemian ja insuliiniresistenssin kanssa. Metabolinen oireyhtymä on näiden poikkeavuuksien kasauma ja tärkeä riskitekijä sekä sydän- ja verisuonitautien että tyypin 2 diabeteksen synnylle. Ruokavalio yhdessä muiden elämäntapamuutosten kanssa on avainasemassa kohonneen verenpaineen ja siihen liittyvien häiriötilojen ehkäisyssä ja hoidossa. Elintarvikkeiden, jotka sisältävät maitoproteiinia peräisin olevia biologisesti aktiivisia laktotripeptidejä, on havaittu laskevan verenpainetta kliinisissä tutkimuksissa. Kaikki julkaistut tutkimukset eivät kuitenkaan ole yhdenmukaisesti osoittaneet verenpainetta alentavaa vaikutusta. Laktotripeptidien vaikutustavan on arveltu perustuvan angiotensiiniä konvertoivan entsyymien estoon.

Lakritsivalmisteiden nauttiminen voi tunnetusti nostaa verenpainetta. Lakritsin sisältämä glykyrritsiini metaboloituu suolistossa glykyrrettiinihapoksi, joka vaikuttaa kortisolin aineenvaihduntaan estämällä 11- β -hydroksisteroidi-dehydrogenaasi-tyyppi-2 (11 β -HSD2) -entsyymiä, joka hajottaa biologisesti aktiivista kortisolia inaktiiviseksi kortisoniksi. Liiallinen lakritsin nauttiminen lisää kortisolin sitoutumista mineralokortikoidireseptoreihin, joita esiintyy useissa kudoksissa kuten munuaisissa, verisuonten seinämissä, sydämessä ja keskushermostossa. Täten mahdollisia lakritsihypertension taustalla olevia mekanismeja ovat solunulkoisen nestetilavuuden kasvu, ääreisverenkierron vastuksen nousu, lisääntynyt valtimojäykkyys tai muutokset sydämen tai autonomisen hermoston toiminnassa.

Tämän väitöskirjatyön tavoitteena oli selvittää reniini-angiotensiini-aldosteroni -järjestelmää mahdollisesti estävien laktotripeptidien sekä kolesterolin imeytymistä estävän kasvisteroliesterin yhteiskäytön vaikutusta hemodynaamiikkaan ja plasman lipideihin henkilöillä, joilla on metabolinen oireyhtymä. Lakritsin aiheuttama verenpaineen nousu on aikaisemmin liitetty solunulkoisen nestetilavuuden lisääntymiseen, joka esiintyy tyypillisesti yhdessä reniini-angiotensiini-aldosteroni -järjestelmän vaimenemisen kanssa. Tämän väitöskirjatyön tavoitteena oli selvittää minkälaiset muutokset verenkiertoelimistön toiminnassa johtavat verenpaineen nousuun lakritsin nauttimisen jälkeen.

Ensimmäisessä osatyössä 104 henkilöä, joilla oli metabolinen oireyhtymä, satunnaistettiin nauttimaan 12 viikon ajaksi hapanmaitovalmistetta, joka sisälsi lakrotripeptidejä joko 5 tai 25 mg/pv yhdessä kasvisteroliesterin (2 g/pv) kanssa, tai hapanmaitovalmistetta ilman tutkittavia yhdisteitä (placebo-valmiste) (I). Osatyö I toteutettiin kaksoissokkoutettuna 3 ryhmän rinnakkaistutkimuksena. Lakritsin vaikutuksia selvittävässä osatöissä (II-IV) verenpaineeltaan normaalit henkilöt (n=22) söivät päivittäin kahden viikon ajan 120-300 g lakritsivalmistetta sisältäen 290-370 mg glykyrritsiiniä vuorokaudessa, ja verrokkeina toimivat henkilöt jatkoivat tavanomaisen ruokavalionsa noudattamista. Verenkiertoelimistön toimintaa tutkittiin kajoamattomalla menetelmällä makuulla ja passiivisen kallistuskokeen aikana käyttäen impedanssikardiografiaa, tonometristä radialispaaineiden mittausta ja pulssiaallon analyysimenetelmää. Lisäksi ensimmäisessä osatyössä verenpainetta seurattiin kotimittausten avulla.

Laktotripeptidejä ja kasvisteroliesteriä sisältävän hapanmaitovalmisteen nauttiminen ei laskenut verenpainetta tai sen osatekijöitä ääreisverenkierron vastusta tai minuuttitilavuutta placebo-valmisteeseen verrattuna henkilöillä, joilla oli metabolinen oireyhtymä. Tulokset olivat yhdenmukaisia sekä laboratorio- että kotimittauksissa. Tutkimusvalmisteen käytön havaittiin kuitenkin laskevan hieman plasman LDL-kolesterolipitoisuutta.

Kahden viikon lakritsialtistus lisäsi solunulkoista nestetilavuutta sekä sentraalista pulssipainetta ja augmentaatioindeksiä osoittaen voimistunutta ääreisverisuonista takaisin heijastuvaa paineaaltoa, ja nosti sekä perifeeristä että sentraalista verenpainetta. Lakritsin nauttimisen havaittiin lisäävän myös ääreisverenkierron vastusta ja suurten valtimoiden jäykkyyttä. Lisäksi osoitettiin, että kallistuskokeen aikana augmentaatioindeksin nousu korostui ja sydämen sykkeen nousu jäi vähäisemmäksi lakritsia nauttineilla henkilöillä. Osatyö IV osoitti, että lakritsin aiheuttama heikentynyt kronotrooppinen vaste pystyasentoon noustessa oli yhteydessä pienentyneeseen virtsan kortisoni:kortisoli -suhteeseen, joka kuvaa 11 β -HSD2-entsyymin estoa. Lisäksi lakritsialtistuksen aiheuttaman ääreisverenkierron vastuksen nousun havaittiin liittyvän heikentyneeseen typpioksidiperäiseen verisuonten laajentumiseen.

Lakritsin aiheuttama verenpaineen nousu ei aiheudu yksinomaan suolan ja nesteen kertymisestä elimistöön vaan vaikutus välittyy usean mekanismin kautta. Verenkiertoelimistön toiminnan mittaaminen pelkästään levossa huomattavasti aliarvioi lakritsin aiheuttamia muutoksia, koska tutkimuksessa havaittu sydämen sykkeen säätelyn muutos ja takaisin heijastuvan paineaallon kasvu korostuivat pystyasennossa.

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ABBREVIATIONS

| | |
|-------------------|-------------------------------------------------|
| 11 β -HSD1 | 11 β -hydroxysteroid dehydrogenase type 1 |
| 11 β -HSD2 | 11 β -hydroxysteroid dehydrogenase type 2 |
| ANOVA | Analysis of variance |
| ACC | American College of Cardiology |
| ACE | Angiotensin-converting enzyme |
| ADI | Acceptable daily intake |
| AHA | American Heart Association |
| AIx | Augmentation index |
| allo-THF+THF | Allo-tetrahydrocortisol plus tetrahydrocortisol |
| Ang | Angiotensin |
| AT1 | Angiotensin II receptor type 1 |
| AT2 | Angiotensin II receptor type 2 |
| BMI | Body mass index |
| BP | Blood pressure |
| CI | Cardiac index |
| CO | Cardiac output |
| DASH | Dietary Approaches to Stop Hypertension |
| EC | Endothelial cell |
| ECG | Electrocardiogram |
| ECW | Extracellular water |
| eNOS | Endothelial nitric oxide synthase |
| ESC | European Society of Cardiology |
| ESH | European Society of Hypertension |
| ET-1 | Endothelin-1 |
| FWA | Forward wave amplitude |
| GR | Glucocorticoid receptor |
| HF | High frequency |
| HR | Heart rate |
| HRV | Heart rate variability |
| ICG _{WB} | Whole body impedance cardiography |

| | |
|-------------------|--------------------------------------|
| IDF | International Diabetes Federation |
| Ile-Pro-Pro | Isoleucine-proline-proline |
| LF | Low frequency |
| LTP | Lactotripeptide |
| MetS | Metabolic syndrome |
| MR | Mineralocorticoid receptor |
| NO | Nitric oxide |
| PGF _{2α} | Prostaglandin F _{2α} |
| PGI ₂ | Prostacyclin |
| PP | Pulse pressure |
| PSe | Plant sterol esters |
| PWV | Pulse wave velocity |
| RAAS | Renin-angiotensin-aldosterone system |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| SHR | Spontaneously hypertensive rats |
| SV | Stroke volume |
| SVR | Systemic vascular resistance |
| SVRI | Systemic vascular resistance index |
| THE | Tetrahydrocortisone |
| TXA ₂ | Thromboxane A ₂ |
| Val-Pro-Pro | Valine-proline-proline |
| VSMC | Vascular smooth muscle cell |

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text using roman numerals I-IV:

- I Hautaniemi EJ, Tikkakoski AJ, Tahvanainen A, Nordhausen K, Kähönen M, Mattsson T, Luhtala S, Turpeinen AM, Niemelä O, Vapaatalo H, Korpela R, Pörsti IH. Effect of fermented milk product containing lactotripeptides and plant sterol esters on haemodynamics in subjects with the metabolic syndrome – a randomised, double-blind, placebo-controlled study. *British Journal of Nutrition* 2015; 114: 376-386.
- II Leskinen MH, Hautaniemi EJ, Tahvanainen AM, Koskela JK, Päälysaho M, Tikkakoski AJ, Kähönen M, Kööbi T, Niemelä O, Mustonen J, Pörsti IH. Daily Liquorice Consumption for Two Weeks Increases Augmentation Index and Central Systolic and Diastolic Blood Pressure. *PLoS One* 2014; 9: e105607.
- III Hautaniemi EJ, Tahvanainen AM, Koskela JK, Tikkakoski AJ, Kähönen M, Uitto M, Sipilä K, Niemelä O, Mustonen J, Pörsti IH. Voluntary liquorice ingestion increases blood pressure via increased volume load, elevated peripheral arterial resistance, and decreased aortic compliance. *Scientific Reports* 2017; 7: 10947.
- IV Hautaniemi EJ, Tikkakoski AJ, Eräranta A, Kähönen M, Hämäläinen E, Turpeinen U, Mustonen J, Pörsti IH. Liquorice ingestion attenuates nitroglycerin-induced vasodilatation and impairs cardiac chronotropic response to upright posture. Submitted.

1 INTRODUCTION

High blood pressure (BP) is a significant preventable risk factor for cardiovascular events and death globally (GBD 2015 Risk Factors Collaborators 2016; Forouzanfar et al. 2017). The metabolic syndrome (MetS) is characterised by elevated BP, abdominal obesity, dyslipidaemia, and insulin resistance (Alberti et al. 2009), and these cardiometabolic risk factors together increase the risk of cardiovascular disease and type 2 diabetes (Ford 2005). Recently, it was estimated that 874 million adults had systolic BP of 140 mmHg or more (Forouzanfar et al. 2017). Thus, the prevention and treatment of this global health problem with both nonpharmacological and pharmacological approaches is essential for reducing the risk of cardiovascular disease and death (Piepoli et al. 2016; Williams et al. 2018). Lifestyle choices including healthy balanced dietary pattern, maintenance of normal body weight, regular physical exercise, and smoking cessation are the cornerstones of the treatment of prehypertension and mild hypertension and other cardiometabolic risk factors, and are also recommended in parallel with pharmacological therapy (Williams et al. 2018).

Milk casein-derived lactotripeptides (LTPs) isoleucine-proline-proline (Ile-Pro-Pro) and valine-proline-proline (Val-Pro-Pro) with possible angiotensin-converting enzyme (ACE) inhibiting properties have been reported to lower BP in several meta-analyses of randomised, placebo-controlled clinical trials (Xu et al. 2008; Cicero et al. 2011a; Qin et al. 2013; Turpeinen et al. 2013; Fekete et al. 2015). However, the antihypertensive efficacy of LTPs has not been consistent in all studies (Engberink et al. 2008; Van Der Zander et al. 2008; Van Mierlo et al. 2009; Usinger et al. 2010b). For the dietary treatment of hypercholesterolemia, the efficacy of food products enriched with phytosterols (plant sterols or stanols) inhibiting the absorption of dietary and endogenous cholesterol is well established (Law 2000; Katan et al. 2003; Abumweis et al. 2008; Demonty et al. 2009; Musa-Veloso et al. 2011; Ras et al. 2014).

Liquorice ingestion may lead to salt and water retention, elevation of BP, hypokalaemia, and suppression of the renin-angiotensin-aldosterone system (RAAS), as firstly described by Conn et al. (1968). Subsequently, liquorice consumption has been widely recognized as a dietary-based reason for elevated BP

(Farese et al. 1991; Sigurjonsdottir et al. 1995; Armanini et al. 1996; Sigurjonsdottir et al. 2001; Sigurjonsdottir et al. 2003). The consumption of liquorice seems to vary among individuals (Simpson and Currie 1982), and in extreme cases the consequences of liquorice exposure may be serious as was demonstrated in a 10-year-old boy who developed a syndrome of posterior reversible encephalopathy (Tassinari et al. 2015).

Liquorice products contain glycyrrhizic acid which is metabolised to glycyrrhetic acid in the bowel (Ploeger et al. 2001). Glycyrrhetic acid inhibits the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) which degrades active cortisol to inactive cortisone (Stewart et al. 1987). In normal conditions, the mineralocorticoid receptor (MR) is occupied by aldosterone (Edwards et al. 1988; Funder et al. 1988), but during inhibition of the enzyme 11 β -HSD2 cortisol binds to the MR (Stewart et al. 1987). The elevation of BP due to liquorice consumption has been associated with sodium retention and water expansion as a consequence of enhanced MR activation by cortisol in the renal tubules. However, the MRs are co-expressed with the 11 β -HSD2 in several other BP regulating tissues including the vascular wall, central nervous system, and the heart (Chapman et al. 2013). Therefore, the liquorice-induced elevation of BP could also be mediated via increased peripheral arterial resistance and large arterial stiffness, or changes in the autonomic tone and cardiac function.

Since the existing data about the antihypertensive effects of LTPs are contradictory, the aim of the present study was to investigate the long-term effects of intake of LTPs previously suggested to inhibit the renin-angiotensin-aldosterone system (RAAS) on haemodynamics in subjects with the MetS. For reduction of total cardiovascular risk, the effective control of risk factors like hypertension and hypercholesterolemia is important (Piepoli et al. 2016). LDL-cholesterol has been reported to directly associate with peripheral and central systolic and diastolic BP (Lamarche et al. 2018), and the level of systemic vascular resistance (SVR) (Choudhary et al. 2018). As parallel lowering of plasma lipids is expected to increase the beneficial cardiovascular effects, the two doses of LTPs (5 and 25 mg/d) were tested in a combination with 2 g/d of plant sterol esters (PSe). In addition, the present investigation examined the detailed haemodynamic alterations leading to the elevation of BP after regular liquorice consumption, which is characterised by the suppression of the RAAS. The influences of two weeks daily liquorice exposure corresponding to intake of 290-370 mg of glycyrrhizin was examined in healthy volunteers. In the present series of studies, a comprehensive haemodynamic recording protocol with passive head-up tilt was utilised.

2 REVIEW OF THE LITERATURE

2.1 Blood pressure

2.1.1 Basic determinants of blood pressure

The blood flow through the circulatory system is equal to cardiac output (CO), the volume of blood that is ejected from the left ventricle of the heart to the aorta during each minute (Guyton and Hall 2011). Because of the pulsatile function of the heart with periods of relaxation (ventricles are filled with blood) and contraction (blood is pushed out of the ventricles) arterial pressure varies between diastolic and systolic pressure. Further, the mean pressure level changes as blood flows through the systemic circulation (Guyton and Hall 2011). Due to the continuous pumping of the blood to the aorta, the pressure is high in the arteries, but decreases in the arterioles and capillaries in the peripheral tissues. Further, when blood returns to the heart through the veins, the mean pressure falls about to zero mmHg in the vena cava (Guyton and Hall 2011). The two factors influencing the blood flow (F) through a vessel are the pressure difference (ΔP) between the opposite ends of the vessel, and the resistance (R) caused by the viscous drag of the blood against the vessel wall, as illustrated by Ohm's law: $F = \Delta P / R$ (Guyton and Hall 2011). Arterial BP is the product of SVR and CO, while heart rate (HR) and stroke volume (SV) are the determinants of CO. SV is influenced by the quantity of blood flowing to the heart i.e. venous return, and contractile force of the heart (Guyton and Hall 2011).

2.1.2 Central wave reflection and arterial stiffness

In spite of the pulsatile function of the heart, the blood flow remains continuous in peripheral capillaries due to the cushioning effect of the compliant artery system (O'Rourke and Hashimoto 2007; Guyton and Hall 2011). The ability of the aorta to dilate in response to increased blood volume during systole, and store the ejected blood volume, maintains the blood flow during diastole (Chemla et al. 2008). The

difference between systolic and diastolic BP equals the pulse pressure (PP) and is affected by SV and arterial compliance (Dart and Kingwell 2001). The components of the aortic pulse wave form are depicted in Figure 1a. The flow ejection from the left ventricle to the aorta induces the forward pressure wave which is integrated with the backward pressure wave reflecting from the sites of peripheral circulation (O'Rourke and Pauca 2004; O'Rourke and Hashimoto 2007; Chemla et al. 2008). Augmentation pressure can be identified as the difference between the first inflection point and the peak of the systolic pressure wave.

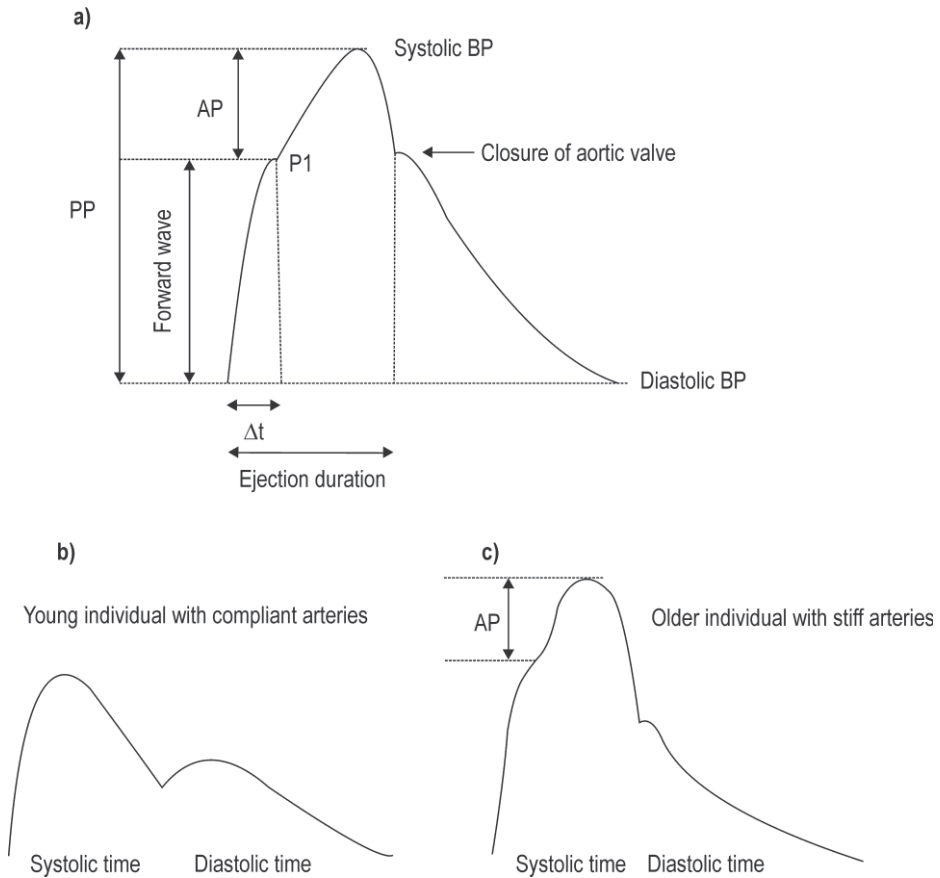


Figure 1. Components of the aortic pulse wave form (a), and the aortic pulse wave form in a young individual with compliant arteries, (b) and in an older individual with stiff arteries (c). AP, augmentation pressure; Δt , time to reflected wave; P1, first inflection point; PP, pulse pressure. (Adapted from O'Rourke and Pauca 2004, and Chemla et al. 2008).

In compliant arteries the reflected wave tends to return to the aortic root during diastole as the pulse wave velocity (PWV) is low (Laurent et al. 2006). Therefore, the augmentation pressure of young individuals is low with only a weak contribution to the systolic BP (Chemla et al. 2008), or the augmentation pressure may be negative (Figure 1b) (O'Rourke and Pauca 2004). In contrast, the stiffening of large arteries (e.g. with aging via changes in the structure of the arterial wall) increases PWV leading to earlier return of the reflected wave, which augments the aortic PP and systolic BP (Figure 1c) (Laurent et al. 2006; O'Rourke and Hashimoto 2007).

The augmentation index (AIx) is the ratio of augmentation pressure to PP, and it is influenced by large arterial stiffening (O'Rourke and Hashimoto 2007). In addition, several other factors have an impact on AIx including height, gender, age, shape and amplitude of the forward wave, duration of the ventricular ejection, SV, HR, and SVR (Kingwell and Gatzka 2002; Laurent et al. 2006; Wilenius et al. 2016). In the assessment of arterial stiffness, the PWV is accepted as the gold standard, while the AIx should only be considered as an index of wave reflection (Laurent et al. 2006). PWV has been shown to independently predict all-cause and cardiovascular mortality in hypertensive subjects (Laurent et al. 2001). In addition, AIx has been proposed to predict future cardiovascular events and all-cause mortality independently of BP (Vlachopoulos et al. 2010).

2.2 Regulation of blood pressure

The basic determinants of arterial pressure, CO and SVR, are continuously regulated via combination of short-term and long-term mechanisms (Guyton and Hall 2011; Navar 2014). The autonomic nervous system is the principal short-term regulator of BP, while the kidneys are the major contributor to the long-term maintenance of BP. In addition, several other systems are involved in the regulation of BP which is controlled by interacting local, neural, humoral and renal factors (Guyton and Hall 2011; Navar 2014).

2.2.1 Autonomic nervous system and kidneys

The autonomic nervous system with a sympathetic and parasympathetic arm regulates BP by controlling HR, cardiac contractility, peripheral vasoconstriction, and renal function via arterial and cardiopulmonary baroreflexes (Cowley et al. 1973;

Grassi et al. 1998; Hart and Charkoudian 2011; Fernandez et al. 2015). The arterial baroreceptors are stretch receptors located in the aortic arch and carotid sinuses, and respond to rapid changes in arterial pressure. They are stimulated when BP is elevated and unloaded when BP falls, which leads to increased or decreased afferent output to the vasomotor centre of the brain stem, respectively, and modulation of the autonomic tone in order to restore the normal pressure level (Guyton and Hall 2011). For example, when BP decreases during orthostatic challenge, the efferent parasympathetic output via vagus nerve to the heart is decreased, while sympathetic output to the heart, vasculature, and kidneys is increased leading to the elevation of BP (Fernandez et al. 2015). The sympathetic signals to the target tissues are mediated via noradrenaline which is released from the local nerve endings. In addition, the adrenal glands release catecholamines, from which about 85 % is adrenaline and 15 % noradrenaline, into the circulation (Guyton and Hall 2011). The effects of noradrenaline and adrenaline are mediated via adrenergic receptors classified as α_1 and α_2 , and β , which are all further divided into subtypes. The α_1 -receptors are important mediators of vasoconstriction (Docherty 2010), while activation of α_2 -receptors can result in increase or decrease in BP depending on the receptor subtype (Philipp et al. 2002). With respect to the β -receptors, β_1 is the predominant receptor increasing HR and cardiac contractility, while β_2 mediates relaxation of smooth muscle in the vasculature and bronchi (Guyton and Hall 2011).

The control of blood volume is essential for the long-term regulation of BP (Navar 2014). The kidneys have an important role in the body fluid homeostasis by modulating the extracellular water (ECW) volume via alterations in fluid and sodium excretion. The elevation of BP is sensed by the kidneys via elevated renal perfusion pressure, which leads to increased water and salt excretion, and subsequent decrease in venous return, CO, and BP (Wadei and Textor 2012). The autonomic nervous system and various hormones contribute to this pressure-natriuresis (Guyton and Hall 2011). The RAAS has a major impact on the rate of salt and water retention (Wadei and Textor 2012).

2.2.2 Renin-angiotensin-aldosterone system

The RAAS is an important regulator of BP via the formation of angiotensin (Ang) II, the major end product that modulates total vascular resistance, and sodium and water balance (Figure 2) (Wadei and Textor 2012; Navar 2014; Te Riet et al. 2015). The RAAS is present both in the circulation and locally in tissues including the

kidneys, adrenal gland, heart, blood vessels, brain, intestine, and adipose tissue (Nguyen Dinh Cat and Touyz 2011; Te Riet et al. 2015). The formation of Ang II is dependent on renin, an aspartyl proteinase that is released from the juxtaglomerular cells of the kidneys into the circulation (Nguyen Dinh Cat and Touyz 2011; Navar 2014; Te Riet et al. 2015). In the blood, renin forms the decapeptide Ang I from the liver-derived angiotensinogen, and the octapeptide Ang II is generated from Ang I by the ACE (Nguyen Dinh Cat and Touyz 2011; Navar 2014). The ACE is present especially on the membrane of the pulmonary endothelial cells (ECs), but is also found in several other tissues. The local RAAS is suggested to contain all the components necessary for Ang II production and the receptors mediating the effects of Ang II (Fyhrquist and Saijonmaa 2008), although the main sites for renin and angiotensinogen formation are the kidneys and liver, respectively (Te Riet et al. 2015). The circulating RAAS regulates short-term cardiovascular-renal homeostasis with the contributing actions of local tissue RAAS (Bader 2010; Nguyen Dinh Cat and Touyz 2011). The local RAAS is also involved in hypertensive and diabetic end-organ damage (Bader 2010).

The release of renin leading to the generation of Ang II is increased when salt intake, renal perfusion pressure, or arterial pressure is decreased, or renal sympathetic nerve activity is increased (Navar 2014). Ang II increases total peripheral resistance via direct constriction of vascular smooth muscle cells (VSMCs) and by enhancing sympathetic nerve activity. In addition, ECW volume is increased through several mechanisms. Ang II stimulates release of aldosterone from the adrenal cortex and acts directly on the proximal tubules further enhancing the aldosterone-induced sodium and water reabsorption (Wadei and Textor 2012). Further, the action of intestinal Ang II increases sodium and fluid absorption from the gut (Navar 2014). As the central mechanisms, Ang II activates the thirst centre in the brain and the hypothalamic-posterior pituitary gland secretory mechanism, which leads to increased intake of fluids and release of arginine vasopressin (antidiuretic hormone), respectively (Guyton and Hall 2011; Navar 2014; Fernandez et al. 2015). In addition, Ang II increases cardiac contractility and CO (Navar 2014).

The inappropriate increase in Ang II level causes hypertension and end-organ damage (Fyhrquist and Saijonmaa 2008). Enhanced action of vascular Ang II induces inflammation, proliferation, and fibrosis in the vessel wall via activation of multiple signalling pathways triggered by generation of reactive oxygen species (ROS) (Te Riet et al. 2015). Also, the components produced by the adipocyte RAAS in the perivascular adventitial tissue might exert detrimental effects on the adjacent vascular

tissue contributing to hypertension and related disorders (Kim et al. 2006; Sarzani et al. 2008; Nguyen Dinh Cat and Touyz 2011).

The effects of Ang II are mediated via two G protein coupled receptors, Ang type 1 (AT1) and Ang type 2 (AT2) (Nguyen Dinh Cat and Touyz 2011; Navar 2014). The major effects of Ang II discussed above are subsequent to the activation of AT1 receptor, while the AT2 receptor is thought to induce opposite actions including vasodilation, excretion of sodium into the urine, and anti-inflammatory, anti-fibrotic, and anti-growth actions (Nguyen Dinh Cat and Touyz 2011). In adults, the expression of AT1 receptors is high, while the AT2 receptors are found at low levels. The latter is the more predominant receptor type in the fetal tissues (Nguyen Dinh Cat and Touyz 2011; Navar 2014).

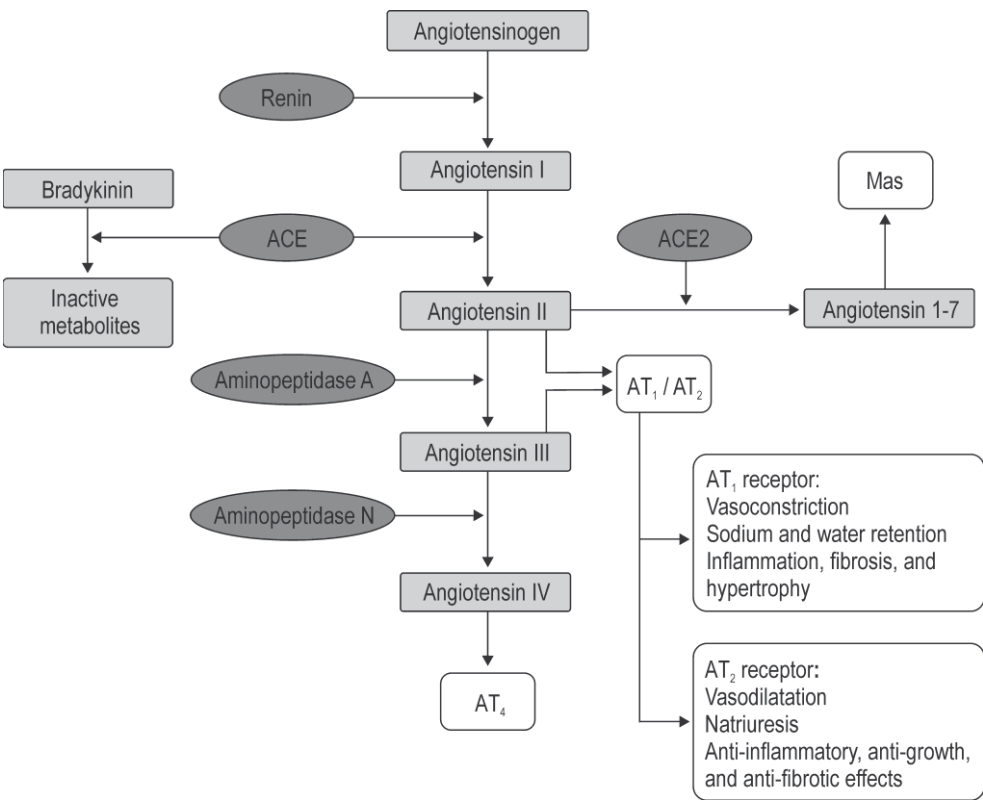


Figure 2. A simplified view of the renin-angiotensin-aldosterone system. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AT₁, angiotensin II receptor type 1; AT₂, angiotensin II receptor type 2; AT₄, angiotensin IV receptor; Mas receptor. (Adapted from Fyhrquist and Saijonmaa 2008, and Nguyen Dinh Cat and Touyz 2011).

In addition to Ang II, the RAAS produces other biologically active peptides including Ang III, Ang IV, and Ang 1-7 (Fyhrquist and Saijonmaa 2008; Nguyen Dinh Cat and Touyz 2011; Te Riet et al. 2015). The Ang III is produced from Ang II by aminopeptidase A, and it possesses similar effects to those of Ang II via AT1 and AT2 receptors. The Ang IV is derived from Ang III by aminopeptidase N and binds to AT4 receptor that is also called the insulin-regulated aminopeptidase. Ang IV induces a wide range of effects including increment in renal blood flow. The Ang 1-7 is generated from Ang II by the ACE2, and exerts opposite actions compared to Ang II (vasodilatation and antiproliferative effects) via the Mas receptor (Fyhrquist and Saijonmaa 2008; Nguyen Dinh Cat and Touyz 2011; Te Riet et al. 2015). Further, the ACE is also connected to the kallikrein-kinin system, as it degrades bradykinin (a vasodilator) to inactive metabolites (Figure 2) (Hillmeister and Persson 2012).

2.2.3 Vascular endothelium

The ECs cover the inner surface of the blood vessels and interact with both the streaming blood and adjacent VSMCs (Rajendran et al. 2013; Zhao et al. 2015; Cahill and Redmond 2016). In response to humoral, neural, and haemodynamic stimuli the endothelium synthesizes and releases various paracrine agents regulating vascular permeability, thrombosis and thrombolysis, platelet and leukocyte adhesion and aggregation, smooth muscle cell growth and migration, and vascular tone. Thus, an appropriate balance and functioning of the endothelium is essential for cardiovascular homeostasis and health (Navar 2014; Cahill and Redmond 2016). The basics of endothelium-mediated regulation of vascular relaxation and constriction are shown in Figure 3.

2.2.3.1 Endothelium-derived vasodilator and vasoconstrictor substances

Nitric oxide (NO) is an important vasodilator agent released from the endothelium to produce relaxation of the VSMCs. The functional existence of this endothelium-derived substance was discovered by Furchgott and Zawadzki (Furchgott and Zawadzki 1980), and subsequently it was identified as NO (Ignarro et al. 1987; Palmer et al. 1987). It is generated from L-arginine by the endothelial NO synthase (eNOS) and when diffused into the VSMC, it stimulates the soluble guanylate cyclase (Zhao et al. 2015). This triggers the formation of cyclic guanosine monophosphate leading to decreased level of intracellular calcium (Ca^{2+}) and subsequent

vasodilatation. Prostacyclin (PGI_2) is another endothelium-derived vasodilating factor causing hyperpolarization of VSMC via increased generation of cyclic adenosine monophosphate through activation of the prostaglandin I_2 (IP) receptor (Feletou et al. 2010; Cahill and Redmond 2016).

In addition to NO and PGI_2 , there are several other pathways and diffusible factors causing endothelium-dependent hyperpolarisation of VSMC (Feletou et al. 2010; Schinzari et al. 2017; Goto et al. 2018). In the EC, increased intracellular Ca^{2+} concentration and subsequent potassium (K^+) outflow via Ca^{2+} -activated K^+ channels induces hyperpolarization, which is transmitted to the adjacent VSMC through myoendothelial gap junctions. Furthermore, elevated K^+ concentration in the intercellular space leads to activation of K^+ channels and Na^+/K^+ -ATPase in the VSMC inducing hyperpolarization, inhibition of Ca^{2+} inflow, and finally vascular relaxation. In addition, several other endothelium-derived factors like epoxyeicosatrienoic acid and hydrogen peroxide can diffuse towards the VSMC and have direct effects on the K^+ channels (Feletou et al. 2010; Schinzari et al. 2017; Goto et al. 2018).

The endothelium also produces vasoconstrictive agents including endothelin-1 (ET-1), Ang II, and prostanoids like thromboxane A_2 (TXA_2), and prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (Kinlay et al. 2001; Feletou et al. 2010; Mackenzie 2011). ET-1 induces its actions via receptors termed ET_A and ET_B , while TXA_2 and $\text{PGF}_{2\alpha}$ mediate vasoconstriction via thromboxane/endoperoxide (TP) receptor and prostaglandin $\text{F}_{2\alpha}$ (FP) receptor, respectively. The subsequent increase in intracellular Ca^{2+} concentration leads to constriction of VSMCs (Vanhoutte et al. 2017).

2.2.3.2 Endothelial dysfunction

Endothelial dysfunction is an imbalance between the production of vasodilator and vasoconstrictor agents with a shift towards decreased release of NO and increased release of prostanoids and ET-1 (Rajendran et al. 2013; Cahill and Redmond 2016; Vanhoutte et al. 2017). In addition to the vasodilatory effects, NO possesses anti-proliferative, antioxidant, and anti-inflammatory properties. With the contribution of PGI_2 , NO inhibits platelet aggregation, leukocyte adhesion, and VSMC proliferation, and limits lipoprotein oxidation. The effects of the vasoconstrictors ET-1 and prostanoids are opposite to those of NO and PGI_2 , and these mediators promote the formation of atherosclerotic plaques when overproduced (Feletou et al. 2010; Rajendran et al. 2013; Cahill and Redmond 2016; Vanhoutte et al. 2017). An important mechanism leading to endothelial dysfunction is an increased production

of ROS resulting in decreased bioavailability of NO. Several factors like smoking, physical inactivity, and obesity are related to increased ROS production (Rajendran et al. 2013; Cahill and Redmond 2016). Further, endothelial dysfunction is associated with several cardiovascular diseases including hypertension, hyperlipidaemia, and diabetes - both as a cause or a consequence (Rajendran et al. 2013; Cahill and Redmond 2016). As an example of medical therapy, ACE-inhibitors and AT₁ receptor antagonist have been shown to beneficially influence endothelial dysfunction (Higashi et al. 2000; Takiguchi et al. 2011). In addition, lifestyle modifications including weight reduction in obese subjects, exercise, and healthy dietary patterns containing vegetables, fruits, fish and nuts may have a beneficial effect on endothelial function (Hamdy et al. 2003; Landberg et al. 2012).

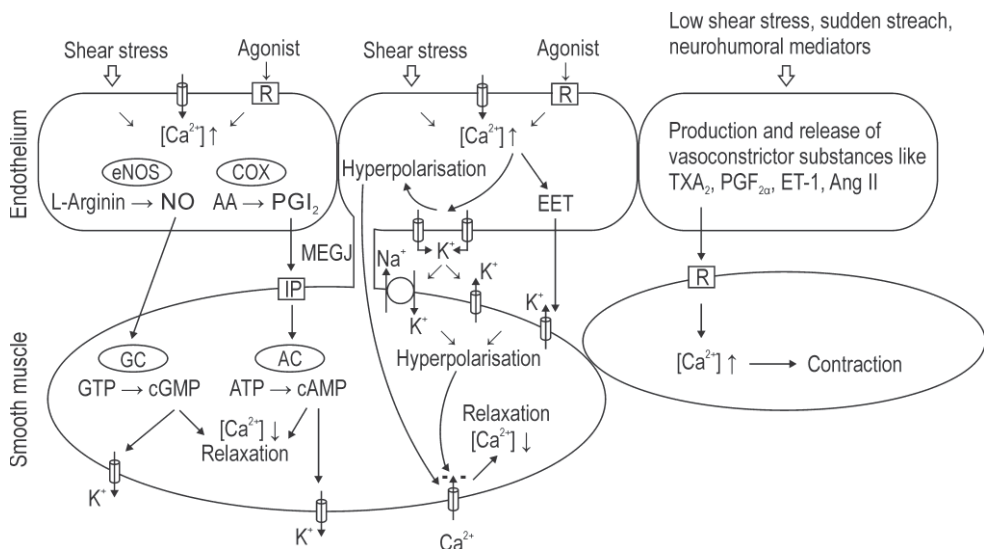


Figure 3. Principal mechanisms of endothelium-mediated relaxation and contraction of vascular smooth muscle. AA=arachidonic acid, AC=adenylate cyclase, Ang II=angiotensin II, ATP=adenosine triphosphate, cAMP=cyclic adenosine monophosphate, cGMP=cyclic guanosine monophosphate, COX=cyclooxygenase, eNOS=endothelial nitric oxide synthase, EET=epoxyeicosatrienoic acid, ET-1=endothelin 1, GC=guanylate cyclase, GTP=guanosine triphosphate, IP=prostaglandin I₂ receptor, MEGJ=myoendothelial gap-junction, NO=nitric oxide, PGF_{2α}=prostaglandin F_{2α}, PGI₂=prostacyclin, R=receptor, TXA₂=thromboxane A₂. (Adapted from Feletou et al. 2010, Cahill and Redmond 2016, Schinzari et al. 2017, Vanhoutte et al. 2017, and Goto et al. 2018).

2.2.4 Glucocorticoids and 11 β -hydroxysteroid dehydrogenase type 2

In humans, adrenal cortex synthesizes cortisol, the main glucocorticoid, in the zona fasciculata-reticularis, and aldosterone, the principal mineralocorticoid, in the zona glomerulosa (Hammer and Stewart 2006; Chapman et al. 2013). The MR has a high affinity for both aldosterone and cortisol (Edwards et al. 1988; Funder et al. 1988), while the glucocorticoid receptor (GR) is selective but has a 10-fold lower affinity for cortisol than the MR (Chapman et al. 2013). Plasma concentrations of cortisol are regulated by the hypothalamic-pituitary-adrenal axis (Hammer and Stewart 2006), while the local glucocorticoid availability and thus action on target tissues is regulated by the type 1 and type 2 isoenzymes of 11 β -HSD (Chapman et al. 2013). The enzyme 11 β -HSD1 catalyses the regeneration of active cortisol from inactive cortisone and enhances the binding of cortisol to the GR. On the contrary, the 11 β -HSD2 degrades cortisol to cortisone and allows only aldosterone to bind to the intracellular MR. Due to this protective effect, aldosterone is the physiological agonist of the MR although it circulates at considerably lower concentrations compared to cortisol (Chapman et al. 2013). The MR and GR are both found in tissues that are involved in the regulation of BP including the kidneys, vascular wall, central nervous system, and the heart (Funder et al. 1989; Goodwin and Geller 2012; Oakley and Cidlowski 2015).

Autosomal recessive inactivating mutations in the 11 β HSD2 gene induce an inherited severe hypertension known as apparent mineralocorticoid excess that leads to sodium and water retention, increased excretion of potassium into the urine, and suppression of the RAAS due to enhanced renal MR activation by cortisol (Mune et al. 1995; Ferrari et al. 1996). The syndrome of apparent mineralocorticoid excess is rare, but mild deficiencies in the function of 11 β HSD2 caused by polymorphisms have been associated with essential hypertension in some patients (Watson et al. 1996; Carvajal et al. 2005; Mariniello et al. 2005), although contradictory data also exist (Kosicka et al. 2013; Mune et al. 2013; Ji et al. 2017). In addition to the altered salt and water balance in the kidneys, elevation of BP can be mediated via vascular mechanisms as the enzyme 11 β -HSD2 has been detected in the vascular wall both in the ECs (Christy et al. 2003) and the VSMCs (Hatakeyama et al. 2000). Deficient activity of 11 β -HSD2 in the vascular wall may increase arterial tone via enhanced contractile responses to pressor hormones and suppression of endothelial NO synthesis (Hadoke et al. 2001; Quaschnig et al. 2001; Ruschitzka et al. 2001; Souness et al. 2002). In the brain, inhibition of 11 β -HSD2 has increased BP in rats (Gomez-Sanchez and Gomez-Sanchez 1992; Zhang et al. 2006). The enzyme 11 β -HSD2 has

been suggested to be present in human cardiac tissue (Lombes et al. 1995), although the expression might be restricted to the coronary vasculature (Chapman et al. 2013). Recent reviews have proposed that activation of cardiomyocyte MR is associated with development and progression of heart disease, while GR signalling may have a role in the maintenance of normal cardiac function in adulthood (Oakley and Cidlowski 2015; Richardson et al. 2016).

2.3 Hypertension and metabolic syndrome

In primary hypertension, which accounts for more than 90 % of the cases, the reason for elevated BP is not known (Oparil et al. 2003). Many factors have been suggested to associate with the development of hypertension, which is considered to belong to the metabolic and cardiovascular diseases (Oparil et al. 2003; Hayden and Sowers 2008). Obesity and insulin resistance are known to increase BP via the contributions of oxidative stress, endothelial dysfunction, inflammation, and increased activity of the RAAS and the sympathetic nervous system (Hayden and Sowers 2008). Hypertensive patients are usually characterised by increased SVR and arterial stiffness, but increased CO may also be involved (Tikkakoski et al. 2013).

According to the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH) the optimal systolic/diastolic BP is less than 120/80, while the normal level of arterial pressure is defined as 120-129/80-84 mmHg (Williams et al. 2018). The grade 1 hypertension is determined as systolic/diastolic BP of 140-159/90-99 mmHg by the ESC/ESH (Williams et al. 2018). However, the American College of Cardiology (ACC) and American Heart Association (AHA) have set the definition of stage 1 hypertension to a lower level of systolic/diastolic BP of 130-139/80-89 mmHg (Whelton et al. 2018).

The International Diabetes Federation (IDF) (Alberti et al. 2005), and the AHA/National Heart, Lung, and Blood Institute (Grundy et al. 2005) have defined diagnostic criteria for the MetS. As a difference between these organizations, an ethnicity specific, increased waist circumference is an obligatory component for MetS in the IDF criteria. Subsequently, a Joint Scientific Statement was published in order to unify criteria for MetS (Alberti et al. 2009). According to this definition, the diagnosis of MetS can be made if 3 abnormal measures out of 5 are found (Table 1).

The global prevalence of hypertension was 31.1 % in 2010, and the proportion of hypertensive adults was higher in low- and middle-income countries (31.5 %) than in high-income countries (28.5 %) (Mills et al. 2016). In Finland, according to the

FinHealth 2017 population study, 48 % of men and 33 % of women aged 30-64 years were hypertensive (Koponen et al. 2018). Hypertension is associated with increased risk of total cardiovascular disease, angina pectoris, myocardial infarction, heart failure, transient ischaemic attack, stroke, and peripheral arterial disease (Rapsomaniki et al. 2014). Furthermore, the MetS is associated with increased risk of all-cause mortality, cardiovascular disease, and type 2 diabetes (Lakka et al. 2002; Ford 2005). The age-adjusted prevalence of MetS in the adult US population was 22.9 % in 2010 (Beltran-Sanchez et al. 2013). In the Finnish population-based Health 2011 Survey, the prevalence of MetS was 54.9 % and 46.4 % in men and women aged 45-54 years, respectively (Koskinen et al. 2012).

The beneficial effect of reduced BP on cardiovascular risk has been shown in clinical trials. A recent meta-analysis of 123 randomised controlled trials of BP lowering medical therapy demonstrated that a 10 mmHg reduction in systolic BP reduced the risk of cardiovascular disease events by 20 %, coronary heart disease by 17 %, stroke by 27 %, and heart failure by 28 % (Ettehad et al. 2016). The treatment of elevated BP aims to the prevention of cardiovascular diseases (Whelton et al. 2018; Williams et al. 2018). A comprehensive treatment strategy including both nonpharmacological and pharmacological therapy is important. The nonpharmacological treatment of hypertension and related disorders is discussed in the next chapter.

Table 1. Diagnostic criteria for the metabolic syndrome according to Alberti et al. (2009). Three abnormal measures out of 5 is required for the diagnosis of metabolic syndrome.

| Measure | Cut point value |
|----------------------------------------------|----------------------------------------------|
| Elevated waist circumference | ≥ 94 cm in men and ≥80 cm in women* |
| Elevated triglycerides [†] | ≥ 1.7 mmol/l |
| Reduced HDL-cholesterol [†] | < 1.0 mmol/l in men and <1.3 mmol/l in women |
| Elevated blood pressure [†] | Systolic ≥ 130 and/or diastolic ≥ 85 mmHg |
| Elevated fasting plasma glucose [†] | ≥ 5.6 mmol/l |

*In European subjects.

[†]Medical therapy for the abnormality in question is an alternative indicator.

2.4 Lifestyle modification in the treatment of hypertension and related disorders

The ESC/ESH and ACC/AHA guidelines emphasize the importance of healthy lifestyle choices in both prevention and treatment of hypertension (Whelton et al. 2018; Williams et al. 2018). In patients with grade 1 hypertension, lifestyle changes may prevent or delay the initiation of medical therapy (Williams et al. 2018). Further,

lifestyle changes in parallel with drug treatment increase the efficacy of antihypertensive pharmacotherapy (Williams et al. 2018). Of note, lifestyle changes are also important in the treatment of other cardiovascular risk factors including the MetS to decrease the risk of cardiovascular diseases (Piepoli et al. 2016). The nonpharmacological treatment includes healthy dietary pattern, weight reduction and maintenance in obese and overweight individuals, regular physical activity, moderation of alcohol intake, and cessation of smoking (Williams et al. 2018).

2.4.1 Nutrition

2.4.1.1 Dietary patterns

The Dietary Approaches to Stop Hypertension (DASH) trial showed that diet rich in vegetables, fruits, and low-fat dairy products, and with reduced amount of total and saturated fat, decreased BP in normotensive and hypertensive subjects in the absence of weight reduction (Appel et al. 1997). In addition, the Mediterranean diet has reduced BP, blood glucose, and total cholesterol, and the incidence of cardiovascular events in subjects with high cardiovascular risk (Estruch et al. 2013; Domenech et al. 2014). Further, adherence to the DASH diet and the Mediterranean diet has been indicated to reduce the risk of type 2 diabetes incidence (Jannasch et al. 2017). The Nordic dietary pattern, an alternative to the Mediterranean diet, has been associated with a beneficial effect on low-grade inflammation and lipid profile in subjects with MetS in a randomized controlled study (SYSDIET) (Uusitupa et al. 2013). Furthermore, in a small SYSDIET sub-study the Nordic diet was found to decrease ambulatory diastolic BP and mean arterial pressure in subjects with MetS without weight reduction (Brader et al. 2014). The Nordic diet was based on the Nordic nutrition recommendations (Nordic Nutrition Recommendations 2004) and included whole-grain products, rich amounts of berries, fruits and vegetables, three fish meals per week, rapeseed oil, and low-fat dairy products, and avoided sugar-sweetened products. Compared to the control diet, fat intake was decreased as saturated fatty acids but increased as polyunsaturated fatty acids, and fibre intake was higher and salt intake lower in the Nordic diet group (Uusitupa et al. 2013; Brader et al. 2014).

Replacement of saturated fatty acids with polyunsaturated fatty acids has been shown to reduce coronary heart disease events (Mozaffarian et al. 2010) and improve glycaemic control, insulin resistance, and insulin secretion capacity (Imamura et al.

2016). In addition, high intake of sugars, especially sugar-sweetened beverages, may have a detrimental effect on cardiovascular risk factors. An intervention study that compared the effect of intake (1 litre/d) of sugar-sweetened soft drink with semi-skimmed milk, water, and aspartame-sweetened drink for 6 months found that consumption of sucrose-sweetened soft drink resulted in ectopic fat deposition in the liver and visceral adipose tissue, and increases in plasma triglycerides and cholesterol compared to the other 3 drinks (Maersk et al. 2012). The consumption of sugar-sweetened beverages has been associated with increased risk of type 2 diabetes (Sonestedt et al. 2012).

The NutriNet-Santé cohort study included 80 426 normotensive French men and women with a mean follow-up of 3.4 years and demonstrated that increased consumption (when comparing the highest to the lowest quartile) of fruit and vegetables, whole grain, nuts, fibre, potassium, magnesium, and plant protein were associated with reduced risk of incident hypertension (Lelong et al. 2017). In contrast, the risk was increased for higher intake of sodium, animal protein, and red and processed meat. Increasingly, scientific literature has implicated that gut microbiota contributes to the regulation of BP (Marques et al. 2018). Further, low bacterial diversity of gut microbiota is known to correlate with overall adiposity, insulin resistance, dyslipidaemia, and low-grade inflammation (Le Chatelier et al. 2013). Diet is a significant modulator of the gut microbiota and factors including high intake of fibre or probiotics may beneficially modify its microbial population (Wen and Duffy 2017; Marques et al. 2018).

2.4.1.2 Milk products

The inverse association of dairy product consumption and risk of hypertension has been proposed in epidemiological studies. A systematic review and meta-analysis of 5 cohort studies including about 45 000 healthy adult subjects and a follow-up range from 2 to 15 years indicated that the highest intake of total dairy foods was associated with a 13 % lower risk of elevated BP when compared with the lowest intake (Ralston et al. 2012). However, comparison of different types of dairy foods showed that reduced risk was found for low-fat and fluid dairy products, while no associations were detected between high-fat dairy foods or cheese and the level of BP (Ralston et al. 2012). These results were in accordance with a subsequent dose-response meta-analysis using data from 9 prospective cohort studies with 57 256 normotensive men and women (Soedamah-Muthu et al. 2012). Results from clinical trials have been inconsistent, as reduction in BP after intake of low-fat milk or dairy

products has been observed in some studies (Buonopane et al. 1992; Van Meijl and Mensink 2011), but not in all (Alonso et al. 2009; Maki et al. 2013). The antihypertensive effect of dairy products has been associated with the intake of calcium, magnesium, potassium (Kris-Etherton et al. 2009), and proteins or bioactive peptides (Korhonen 2009; Pal and Ellis 2010; Fekete et al. 2016).

Milk and dairy product consumption have been suggested to associate with reduced risk of MetS and type 2 diabetes, and not to increase the risk of cardiovascular disease (Lovegrove and Givens 2016). In addition to BP, the effect of dairy product intake on other metabolic risk factors has been studied in observational and in some intervention studies (Astrup 2014). In three cohort studies including 120 877 men and women, yoghurt consumption had a protective effect against weight gain within each 4-year period during 20 years of follow-up (Mozaffarian et al. 2011). In two meta-analyses of randomized controlled trials, intake of dairy products induced significantly beneficial effects on body weight and composition only during simultaneous energy restriction (Abargouei et al. 2012) or in short-term studies (Chen et al. 2012).

Dairy products are sources of saturated fatty acids including lauric, palmitic, and myristic acid which increase serum LDL-cholesterol (Mensink et al. 2003). In an intervention study that investigated the effects of high versus low dietary calcium intake combined with high fat and low fat diet, LDL-cholesterol was found to increase with increased intake of dairy fat (Lorenzen and Astrup 2011). However, according to recent reviews not all studies have detected a significant hypercholesterolaemic effect for dairy product consumption (Huth and Park 2012; Lovegrove and Givens 2016). A meta-analysis of controlled studies with healthy individuals randomised to increased dairy product intake (mean +3.6 servings/d) without other dietary interventions proposed that increase in low fat or high fat milk product intake induces no significant change in LDL-cholesterol concentration as the mean differences were -1.42 mg/dl (95 % CI -4.74 to 1.91 mg/dl) and 3.30 mg/dl (95 % CI -4.30 to 10.90 mg/dl), respectively (Benatar et al. 2013). However, consumption of whole milk has elevated total and LDL-cholesterol compared with low-fat milk as reviewed by Huth and Park (2012). A meta-analysis of randomised controlled trials that examined the effect of cheese consumption on plasma lipids in healthy adults found that cheese intake increased total and LDL-cholesterol compared with tofu and fat-modified cheese, but decreased LDL-cholesterol and HDL-cholesterol when compared with butter intake of similar ratio of polyunsaturated fatty acids to saturated fatty acids (De Goede et al. 2015). Milk contains other ingredients including calcium and proteins, which may modify the

effects on plasma lipids (Bjornshave and Hermansen 2014). In two small intervention studies dairy calcium attenuated the increase in total and LDL-cholesterol concentrations induced by diet rich in dairy fat (Lorenzen and Astrup 2011; Soerensen et al. 2014). In addition, probiotic fermented milk products have been indicated to decrease plasma total and LDL-cholesterol levels (Shimizu et al. 2015). Finally, analysis of data from three prospective cohort studies indicated that replacement of dairy fat with vegetable fat and polyunsaturated fatty acids is associated with decreased risk of cardiovascular disease (Chen et al. 2016).

There is some evidence suggesting that milk product intake might have a beneficial impact on insulin resistance although the number of clinical trials is quite small (Lovegrove and Givens 2016). For example, in a randomized cross-over study, low-fat milk product consumption (4 servings/d) for 6 months had no effect on plasma glucose but decreased plasma insulin and improved homeostatic model assessment for insulin resistance when compared with limited dairy intake of no more than 2 servings/d (Rideout et al. 2013). With respect to low-grade inflammation, a meta-analysis of 8 randomised controlled trials implicated that dairy product intake induced no adverse effects on biomarkers of inflammation in overweight or obese adults (Labonte et al. 2013). However, due to methodologic factors and limitations among the studies the authors were not able to conclude whether the effect of dairy product consumption on inflammation is beneficial or neutral (Labonte et al. 2013).

2.4.1.3 Dietary salt

The INTERSALT study demonstrated a significant relationship between sodium intake and BP (Intersalt Cooperative Research Group 1988). According to a recent meta-analysis, a salt reduction of about 4.4 g/d decreased systolic/diastolic BP by 2.42/1.00 mmHg and 5.39/2.82 mmHg in normotensive and hypertensive subjects, respectively (He et al. 2013). Further, reduced dietary salt intake has been shown to decrease BP dose-dependently in normotensive and hypertensive subjects during the DASH diet, and in hypertensive subjects the low intake of salt (3.75 g/d) combined with the DASH diet reduced systolic BP by 11.5 mmHg (Sacks et al. 2001). However, currently the optimal range of sodium intake is debated as cohort studies have indicated controversial findings on the relationship between dietary sodium intake and cardiovascular risk. Results from the Trials of Hypertension Prevention demonstrated that sodium intake was linearly associated with a risk of cardiovascular events and suggested benefits for reduced sodium intake of 1.5 to 2.3 g/d (3.8 to 5.8

g of salt per day) (Cook et al. 2014). However, there are also reports of a U-shaped relationship (O'Donnell et al. 2014; Mente et al. 2016). For example, Mente et al. (2016) reported that high sodium intake of >6 g/d (15.2 g/d of salt) was associated with an increased risk of cardiovascular events and death only in hypertensive subjects, while lower sodium intake of <3 g/d (7.6 g/d of salt) was associated with increased risk in both normotensive and hypertensive individuals when compared with sodium intake of 4-5 g/d (10.2-12.7 g/d of salt). Currently, the guidelines for the management of hypertension recommend to limit the intake of salt to below 5 g/d (Williams et al. 2018).

2.4.1.4 Plant sterols

Phytosterols (plant sterols and plant stanols) have a structure of steroid alkaloids and are found in vegetable food so that the mean intake from the Finnish diet is about 200-300 mg/d (Valsta et al. 2004). The cholesterol-lowering effect of food products enriched with phytosterols was demonstrated first by the use of plant stanol ester margarine in mildly hypercholesterolemic subjects (Miettinen et al. 1995). Subsequently, reduced plasma cholesterol and LDL-cholesterol levels have been shown after intake of different food formats enriched with phytosterols including low-fat milk products (Law 2000; Katan et al. 2003; Abumweis et al. 2008; Demonty et al. 2009; Musa-Veloso et al. 2011; Ras et al. 2014). In a recent meta-analysis, phytosterol dose of about 2 g/d lowered LDL-cholesterol levels by 8-10 % (Ras et al. 2014). Further, doses ranging from 0.6 to 3.3 g/d were observed to reduce LDL-cholesterol in a dose-dependent fashion by about 6-12 %. Also, phytosterols have been demonstrated to lower serum triglyceride concentration if the baseline levels are high (Naumann et al. 2008; Demonty et al. 2013). The mechanism of action for phytosterols include inhibition of absorption of both dietary and endogenous cholesterol (De Smet et al. 2012). The cholesterol-lowering efficacy of phytosterols has been shown in subjects with MetS (Plat et al. 2009; Sialvera et al. 2012) and type 2 diabetes (Gylling and Miettinen 1994; Baker et al. 2009). According to the European Atherosclerosis Society Consensus Panel, food products enriched with phytosterols can be used as an adjunct to lifestyle interventions or lipid-lowering therapy in individuals who fail to achieve LDL-cholesterol targets on statins or have statin intolerance (Gylling et al. 2014).

2.4.2 Other lifestyle factors

Weight reduction has been shown to reduce BP and the incidence of type 2 diabetes and cardiovascular disease (Tuomilehto et al. 2001; Gregg et al. 2016). In a recent meta-analysis, weight reduction of 5-10 % was detected to lower systolic and diastolic BP by 4.9 and 2.6 mmHg, respectively, over a period of 6 to 12 months (Zomer et al. 2016). Furthermore, epidemiological data has indicated a strong relationship between alcohol consumption and elevated BP (Klatsky 2002). In addition, heavy alcohol intake is associated with increased risk of stroke (Zhang et al. 2014). Reduction of heavy drinking has been shown to reduce BP; in a meta-analysis by Xin et al. self-reported reduction in alcohol consumption (range from 16 % to 100 %, median of 76 %) was associated with decreased systolic and diastolic BP of -3.31 and -2.04 mmHg, respectively (Xin et al. 2001). According to the ESC/ESH guidelines, hypertensive male subjects are recommended to limit their alcohol consumption to <14 units/week and female subjects to <8 units/week (Williams et al. 2018).

Smoking is a major cardiovascular risk factor and cessation of smoking has a high importance in the prevention of cardiovascular disease (Williams et al. 2018). The awake ambulatory systolic BP has been shown to be higher in untreated hypertensive smokers than non-smoking hypertensives although the office BP measurements were same (Mann et al. 1991). In addition, physical activity, exercise training, and overall cardiorespiratory fitness are known to contribute to the prevention and treatment of cardiovascular disease (Lavie et al. 2015). A meta-analysis of different forms of exercise training demonstrated the efficacy of exercise, albeit rather moderate, in reduction of systolic and diastolic BP (Cornelissen and Smart 2013).

2.5 Milk casein-derived lactotripeptides Ile-Pro-Pro and Val-Pro-Pro

Milk is an important source of bioactive peptides that can be released from dairy proteins, whey and casein, during 1) gastrointestinal digestion by digestive or gut microbial enzymes, 2) food processing, and 3) hydrolysis by enzymes isolated from microorganisms (Korhonen 2009; Marcone et al. 2017). Several in vitro and in vivo studies have demonstrated various health benefits for both whey- and casein-derived peptide fragments including antihypertensive, antithrombotic, anti-oxidative, anti-inflammatory, antimicrobial, immunomodulatory, antilipaemic and insulinotropic effects during the last two decades, as reviewed recently (Marcone et al. 2017). The

casein-derived Ile-Pro-Pro and Val-Pro-Pro are the most widely studied LTPs for possible antihypertensive actions (Fekete et al. 2013). Ile-Pro-Pro and Val-Pro-Pro with ACE-inhibitory properties have been detected from sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura et al. 1995a; Nakamura et al. 1995b; Nakamura et al. 1996). Also, Ile-Pro-Pro and Val-Pro-Pro have been produced by enzymatic hydrolysis using an *Aspergillus oryzae* protease (Mizuno et al. 2004; Mizuno et al. 2005).

2.5.1 Influence on blood pressure

2.5.1.1 Animal studies

The acute BP-lowering effect of Ile-Pro-Pro and Val-Pro-Pro has been demonstrated after 6 hours of a single oral administration in spontaneously hypertensive rats (SHR) but not in normotensive Wistar-Kyoto rats (Nakamura et al. 1995b; Masuda et al. 1996). In addition, long-term intake of Ile-Pro-Pro and Val-Pro-Pro in water, or in fermented milk product, has attenuated the development of hypertension in SHR (Nakamura et al. 1996; Sipola et al. 2001; Sipola et al. 2002a; Jauhiainen et al. 2005a; Jäkälä et al. 2009b), salt-loaded type 2 diabetic Goto-Kakizaki rats (Jäkälä et al. 2009a), and double transgenic rats with malignant hypertension (Jauhiainen et al. 2010a). In these studies the treatment period ranged from 8 to 16 weeks, and the antihypertensive effect was more pronounced for fermented milk products than water containing Ile-Pro-Pro and Val-Pro-Pro. Furthermore, long-term feeding of SHR with fermented milk product enriched with LTPs and plant sterols has decreased BP in established hypertension (Ehlers et al. 2011).

2.5.1.2 Clinical studies

The effects of Ile-Pro-Pro and Val-Pro-Pro on BP have been investigated in several controlled clinical trials. Results from meta-analyses have shown reductions of 4-4.8/1.9-2.2 mmHg (Xu et al. 2008; Turpeinen et al. 2013), 3.73/1.97 mmHg (Cicero et al. 2011a), 1.66/0.76 mmHg (Qin et al. 2013), and 2.95/1.51 mmHg (Fekete et al. 2015) in systolic/diastolic BP, respectively, after consumption of LTPs. Although these meta-analyses indicated statistically significant antihypertensive effects for Ile-Pro-Pro and Val-Pro-Pro, not all clinical trials have been able to demonstrate this

(Engberink et al. 2008; Van Der Zander et al. 2008; Van Mierlo et al. 2009; Usinger et al. 2010b).

The conducted studies have used a wide range of doses (from 2.6 to 52.5 mg/d) of LTPs during intervention periods lasting from 4 to 21 weeks. The LTPs have been produced by bacterial fermentation and enzymatic hydrolysis, and the food matrices delivering LTPs have included milk products (Hata et al. 1996; Jauhiainen et al. 2005b; Engberink et al. 2008; Van Der Zander et al. 2008; Van Mierlo et al. 2009; Jauhiainen et al. 2010b), tablets (Aihara et al. 2005; Mizuno et al. 2005), capsules (Hirota et al. 2007; Boelsma and Kloek 2010), fruit- and vegetable juices (Sano et al. 2005; Cicero et al. 2010), and spread (Turpeinen et al. 2009; Turpeinen et al. 2012). The effects of Ile-Pro-Pro and Val-Pro-Pro have been investigated in European (Cicero et al. 2013) and Japanese subjects (Chanson-Rolle et al. 2015), and one study was conducted in the USA (Germino et al. 2010). Most of the trials have included prehypertensive and hypertensive subjects and investigated the effect of LTPs on office BP, although some studies have also examined 24-h ambulatory and home BP measurements.

The available data suggests that ethnic factors may influence the antihypertensive effect of LTPs, as the reduction in systolic/diastolic BP has been more pronounced in the Asian (-5.63/-2.58 mmHg) than European (-1.28/0.59 mmHg) subjects, respectively (Cicero et al. 2013; Chanson-Rolle et al. 2015). In addition, baseline BP and duration of intervention period may matter, as the treatment effects have been greater in hypertensive than normotensive and prehypertensive subjects (Fekete et al. 2015), and according to some meta-analyses, during longer periods of >4 to 8 weeks (Xu et al. 2008; Qin et al. 2013). A few studies have proposed a slight dose-dependent effect of LTPs on office systolic BP (Mizuno et al. 2005; De Leeuw et al. 2009), although meta-analyses have not observed benefits for increasing doses (Cicero et al. 2011a; Chanson-Rolle et al. 2015; Fekete et al. 2015). In some studies using office, 24-h ambulatory, and home BP measurements the BP-lowering effect of LTPs was not consistently observed in all performed recordings (De Leeuw et al. 2009; Cicero et al. 2011b; Cicero et al. 2012; Turpeinen et al. 2012). Further, some meta-analyses have indicated numerically more pronounced BP-lowering effect for fermented than enzymatically produced LTPs (Qin et al. 2013; Fekete et al. 2015). Different production methods (bacterial fermentation or enzymatic hydrolysis) might result in divergent structural properties of LTPs (*cis/trans* configurations) influencing the possible ACE-inhibitory efficacy of these compounds (Siltari et al. 2014).

2.5.2 Mechanisms of possible antihypertensive effects and bioavailability of lactotripeptides

The BP-lowering action of LTPs has been suggested to result from inhibition of the ACE. Several milk-derived peptides have demonstrated ACE-inhibitory activity *in vitro*, but the antihypertensive effect might be absent *in vivo* due to gastrointestinal breakdown of these peptides (Hernandez-Ledesma et al. 2011; Miner-Williams et al. 2014; Marcone et al. 2017). Ile-Pro-Pro and Val-Pro-Pro have been shown to possess ACE-inhibitory activity with IC₅₀ values of 5 μ M and 9 μ M, respectively (Nakamura et al. 1995a). The IC₅₀ refers to the concentration inhibiting the ACE activity by 50 % *in vitro*. Some *in vivo* experiments have supported the concept of ACE inhibition by Ile-Pro-Pro and Val-Pro-Pro in animals, as plasma renin activity was increased (Sipola et al. 2002a), and ACE activity of the aorta was reduced (Nakamura et al. 1996) in SHR after long-term intake of these tripeptides. Also, long-term intake of milk product containing LTPs and PSe decreased serum ACE-activity in SHR (Jäkälä et al. 2009b) and salt-loaded type 2 diabetic Goto-Kakizaki rats (Jäkälä et al. 2009a). However, the ACE inhibitory activity of LTPs has not been proven in humans (De Leeuw et al. 2009; Wuerzner et al. 2009; Boelsma and Klock 2010; Usinger et al. 2010a).

The bioavailability of bioactive peptides has been debated in the scientific literature. A recent review suggested that intact absorption of large peptides from the intestinal lumen into the hepatic portal system is poor due to hydrolyzing peptidases in the enterocytic brush border membrane and cytosol (Miner-Williams et al. 2014). Furthermore, subsequent breakdown of peptides may occur by vascular endothelial tissue and soluble plasma peptidases (Miner-Williams et al. 2014). However, some di- and tripeptides have been suggested to pass intact across the intestinal epithelium and reach the circulation. One proposed absorption mechanism for LTPs is paracellular diffusion (Satake et al. 2002). Ile-Pro-Pro has been detected in human plasma after ingestion of LTP-enriched yoghurt beverage in lower (nanomolar) concentrations than IC₅₀ value of this tripeptide (Foltz et al. 2007). Thus, other properties of LTP might contribute to their antihypertensive effects, including activation of opioid receptors in the gastrointestinal tract (Hernandez-Ledesma et al. 2011; Miner-Williams et al. 2014). A milk-derived tetrapeptide, α -lactorphin, lowered BP in SHR, and this effect was reversed by a specific opioid receptor antagonist (Nurminen et al. 2000). Subsequently, α -lactorphin was proposed to improve endothelium-dependent vascular relaxation via NO release (Sipola et al. 2002b). Furthermore, Miner-Williams et al. (2014) suggested that

inhibition of local intestinal renin-angiotensin system by LTPs might lower BP via modulation of water and sodium absorption from the gut.

2.5.3 Influence on vascular function, arterial stiffness, and wave reflections

The effects of LTPs on endothelial function have been investigated in experimental and clinical studies. For example, acetylcholine-induced endothelium-dependent relaxation was improved in the mesenteric arteries of SHR after long-term treatment with milk containing LTPs and PSe via NO- and endothelium-derived hyperpolarising factor-dependent mechanisms (Ehlers et al. 2011). Also, a Japanese research group observed LTP-induced endothelium-dependent relaxation in isolated aortic rings of Wistar rats, the effect of which was inhibited by eNOS inhibitor, bradykinin receptor antagonist, and K⁺-channel inhibitor (Hirota et al. 2011). In clinical studies, ingestion of casein hydrolysate containing Ile-Pro-Pro and Val-Pro-Pro for one week increased the maximum forearm blood flow during reactive hyperemia indicating improved vascular endothelial function, although no changes were observed in systemic BP (Hirota et al. 2007). Furthermore, consumption of tablets containing LTPs for 4 weeks ameliorated the square root of the ratio of peak:baseline pulse volume during hyperaemia, another variable of endothelial function, in subjects with MetS (Cicero et al. 2016). However, intake of fermented milk product containing LTPs had no effect on AIx responses to nitroglycerin and salbutamol after 12 weeks of intervention (Jauhiainen et al. 2010b).

With respect to indices of arterial stiffness and wave reflections, the effect of Ile-Pro-Pro and Val-Pro-Pro has been investigated on ambulatory arterial stiffness index (Jauhiainen et al. 2007), carotid arterial compliance (Yoshizawa et al. 2009), PWV (Turpeinen et al. 2009; Cicero et al. 2011b; Cicero et al. 2016), and AIx (Nakamura et al. 2009; Turpeinen et al. 2009; Jauhiainen et al. 2010b). These clinical studies, except for the study of Turpeinen et al. (2009), showed statistically significant improvements in the above-mentioned variables after intake of LTPs for 4 to 10 weeks. However, the study of Nakamura et al. (2009) was quite small and without controls. In the study of Turpeinen et al. (2009) the LTPs were incorporated into spread also containing plant sterols. The investigation of Yoshizawa et al. (2009) included postmenopausal women, in which the LTP-induced improvement in carotid arterial compliance was associated with reduced arterial BP, and numerically but non-significantly decreased plasma Ang II levels.

2.6 Liquorice

The commercial liquorice is obtained mainly from the dried root of *Glycyrrhiza glabra*, although the genus *Glycyrrhiza* comprises about 30 species (Isbrucker and Burdock 2006; Omar et al. 2012; Nazari et al. 2017). Liquorice is cultivated in southern Europe, Russia, China, Iran, Iraq, and Turkey, and liquorice extract is commonly used as a sweetener or flavouring agent. In addition to liquorice in candy, it is found in many products including confectionary, chewing gum, non-alcoholic and alcoholic drinks, herbal tea, chewing tobacco, herbal medicines and medicinal preparations like cough medicine (Armanini et al. 2003). A mean daily intake of glycyrrhizin of 1 mg has been estimated for the United Kingdom (Spinks and Fenwick 1990) and a range of 1.6-215 mg/d or 0.027-3.6 mg/kg/d for the United States (Isbrucker and Burdock 2006). According to one survey among 603 high school students in New Zealand, 29 % of the girls and 17 % of the boys consumed liquorice on a weekly basis (Simpson and Currie 1982). Furthermore, 5.9 % and 4.9 % of the girls and boys, respectively, consumed at least 200 g of black liquorice sweet weekly, and 1.8 % and 1 % of the girls and boys, respectively, at least 500 g weekly. Consumption of over 1000 g per week was reported in two students. Thus, the consumption of glycyrrhizin and liquorice seems to vary greatly among populations. Excessive liquorice consumption is often accompanied by elevation of BP (Nazari et al. 2017), and may be one explanation for treatment resistant hypertension (Rossi et al. 2011).

2.6.1 Active constituents of liquorice - structure and metabolism

Liquorice root contains many ingredients of which glycyrrhizin is the most important and appears as potassium and calcium salts of glycyrrhizic acid (Isbrucker and Burdock 2006; Omar et al. 2012; Nazari et al. 2017). The chemical structure of this triterpenoid saponin is composed of glycyrrhetic acid that is conjugated to two molecules of glucuronic acid. The structures of glycyrrhizin (glycyrrhizic acid) and glycyrrhetic acid are presented in Figure 4. The oral bioavailability of glycyrrhizic acid is poor and it is absorbed as a glycyrrhetic acid following hydrolysis by specialized β -glucuronidase of intestinal bacteria (Ploeger et al. 2001). After hepatic uptake by capacity-limited carriers, glycyrrhetic acid is further metabolised to glucuronide and sulphate conjugates which are transported into bile and excreted into the duodenum (Ploeger et al. 2000b). Subsequently, these conjugates are

hydrolysed to glycyrrhetic acid by commensal bacteria leading to reabsorption of this active metabolite of liquorice. Enterohepatic cycling and storage of glycyrrhetic acid conjugates in the gallbladder induces several plasma concentration peaks of glycyrrhetic acid and might lead to accumulation when liquorice products are consumed regularly (Ploeger et al. 2000a; Ploeger et al. 2000b). The degree of reabsorbed glycyrrhetic acid will depend on the transit time of gastrointestinal contents through the bowels (Ploeger et al. 2001).

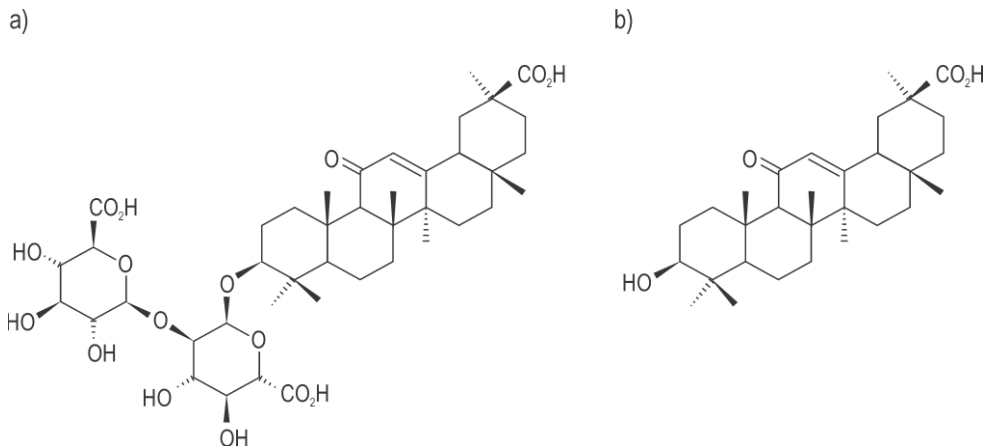


Figure 4. Chemical structure of glycyrrhizin (a) and glycyrrhetic acid (b). (Adapted from Isbrucker and Burdock 2006).

2.6.2 Effects of liquorice consumption

2.6.2.1 Pseudohyperaldosteronism and glucocorticoids

Sodium and water retention, elevation of BP, hypokalaemia, and suppression of the RAAS are well known consequences of liquorice ingestion (Conn et al. 1968; Farese et al. 1991; Armanini et al. 1996). This syndrome is known as pseudohyperaldosteronism due to low plasma aldosterone concentration and low renin activity, first described by Conn et al. (1968) and subsequently by many other authors (Epstein et al. 1977a; Forslund et al. 1989; Farese et al. 1991; Kageyama et al. 1992; Bernardi et al. 1994; Armanini et al. 1996). Altered cortisol metabolism leads to increased urinary excretion of free cortisol and decreased levels of both plasma cortisone and urinary excretion of free cortisone after glycyrrhizin or pure

glycyrrhetic acid ingestion in healthy subjects (Mackenzie et al. 1990; Kageyama et al. 1992). Although the plasma half-life of cortisol is prolonged, plasma cortisol concentration and the function of pituitary-adrenal axis remain unchanged (Epstein et al. 1978; Kageyama et al. 1992). In addition, the urinary ratio of cortisone to cortisol metabolites, that is the ratio of tetrahydrocortisone (THE) to allo-tetrahydrocortisol plus tetrahydrocortisol (allo-THF+THF), is decreased (Farese et al. 1991), or the ratio of cortisol to cortisone metabolites is increased (Armanini et al. 1996; Ferrari et al. 2001).

Evidence about the mineralocorticoid-like effect of liquorice derivatives has been published (Armanini et al. 1996, Calo et al. 2004), and direct binding to the MR was originally considered as the mechanism of the liquorice-induced effects. However, the affinity of liquorice derivatives to the MR is considerably lower than that of aldosterone (Armanini et al. 1983, Takeda et al. 1987). Subsequently, the inhibition of the enzyme 11 β HSD2 with the following enhanced activation of the MR and GR by endogenous cortisol was found to produce the clinical symptoms of liquorice ingestion (Stewart et al. 1987; Whorwood et al. 1993). Of note, the active metabolite of liquorice, glycyrrhetic acid, is a 200-1000 times more potent inhibitor of 11 β HSD2 than glycyrrhizic acid *in vitro* (Buhler et al. 1991, Ploeger et al. 2001). Liquorice derivatives also inhibit the enzyme 11 β HSD1. However, the natural glycyrrhetic acid has been demonstrated to preferentially inhibit the 11 β HSD2, while its diastereomeric derivative acts on the 11 β HSD1 (Classen-Houben et al. 2009). Furthermore, glycyrrhetic acid may also suppress hepatic aldosterone catabolism via inhibition of 5 β -reductase and 3 β -hydroxysteroid dehydrogenase (Latif et al. 1990).

The liquorice-induced metabolic alterations can be detected within a week after liquorice consumption of 100-200 g/d (Epstein et al. 1977a). Furthermore, after cessation of liquorice ingestion the normalization of urinary cortisol excretion and plasma potassium can take 1-2 weeks (Epstein et al. 1977a; Epstein et al. 1977b; Epstein et al. 1978). After prolonged regular liquorice intake of 6 months to 5 years with daily doses of 25-200 g, the suppression of RAAS may sustain for 2-4 months (Epstein et al. 1977b; Farese et al. 1991).

2.6.2.2 Hypertension

The elevation of BP after ingestion of commercial liquorice product or pure glycyrrhizic or glycyrrhetic acid has been shown in clinical studies (Sigurjonsdottir et al. 1995; Armanini et al. 1996; Van Gelderen et al. 2000; Ferrari et al. 2001; Sigurjonsdottir et al. 2001; Sigurjonsdottir et al. 2003), and animal models (Kageyama

et al. 1994; Rossi et al. 1994; Ruszymah et al. 1995; Quaschnig et al. 2001; Ruschitzka et al. 2001). In humans, liquorice intake of 50-200 g/day, corresponding to daily intake of 75-540 mg of glycyrrhetic acid for 2-4 weeks elevated systolic BP by 3.1-14.4 mmHg in a linear dose-response manner (Sigurjonsdottir et al. 2001). However, some clinical studies have indicated no significant influences on BP after pure liquorice extract, glycyrrhetic acid or glycyrrhizin salt tablets in healthy subjects (Whitworth et al. 1994; Armanini et al. 2003; Mattarello et al. 2006; Sobieszczyk et al. 2010; Tu et al. 2010; Yan et al. 2013). In these studies, the daily dose of glycyrrhizin/glycyrrhizic acid or glycyrrhetic acid ranged from 75 to 300 mg with treatment periods of 5 days to 2 months. A recent meta-analysis suggested a significant increase of 5.45/3.19 mmHg in systolic/diastolic BP after regular ingestion of glycyrrhizic acid (Penninkilampi et al. 2017). Also, a dose-response relationship between glycyrrhizic acid and change in systolic BP ($r^2=0.55$, $p=0.04$) and diastolic BP ($r^2=0.65$, $p=0.01$) was detected (Penninkilampi et al. 2017).

2.6.2.3 Impact of dose and individual sensitivity on side effects

A regular daily intake of 100 mg glycyrrhizic acid has been estimated to cause adverse effects in sensitive individuals, while the daily consumption of 400 mg leads to harmful effects in most subjects (Stormer et al. 1993). The European Commission Scientific Committee on Food (2003) and the World Health Organization Expert Committee (2005) have considered 100 mg/day as the upper limit for regular glycyrrhizic acid intake to protect majority of adult population against adverse effects (European Commission 2003; World Health Organization 2005). However, both of these committees concluded that the human data were inadequate to derive an acceptable daily intake (ADI) for glycyrrhizic acid. Some individuals may experience adverse effects even with smaller ingested doses of glycyrrhizic acid when compared with the doses that are expected to induce toxicity. For example, according to one case report a consumption of chewing gum flavoured with liquorice (dose of glycyrrhizic acid 50 mg/d) induced hypokalaemia, oedema, and elevation of BP in a young female (De Klerk et al. 1997). Further, liquorice-induced effects including changes in plasma electrolytes and urinary cortisol/cortisone excretion may be present without elevation of BP (Sobieszczyk et al. 2010), while some individuals do not develop hypokalaemia after liquorice exposure (Armanini et al. 2003). The variability of glycyrrhizin content among different liquorice products (Spinks and Fenwick 1990; Blomberg and Hallikainen 1993) and individual susceptibility to liquorice makes the estimation of the dose-effect level difficult. In addition, oral

intake of liquorice root extract containing glycyrrhizic acid has resulted in lower bioavailability of glycyrrhetic acid when compared to administration of pure glycyrrhizic acid (Cantelli-Forti et al. 1994; Wang et al. 1995). However, ADI of 0.2 mg/kg/d (Van Gelderen et al. 2000) and 0.015-0.229 mg/kg/d (Isbrucker and Burdock 2006) has been proposed for glycyrrhizic acid.

The reported factors to increase sensitivity to glycyrrhizin include hypertension, hypokalaemia, female sex, old age, anorexia nervosa and prolonged gastrointestinal transit time (Omar et al. 2012; Nazari et al. 2017). The daily exposure of 150 mg glycyrrhetic acid for 4 weeks elevated systolic/diastolic BP by 3.5/3.6 mmHg in the normotensive versus 15.3/9.2 mmHg in hypertensive subjects (Sigurjonsdottir et al. 2003). In addition, a more marked increase has been detected in both mean BP and the urinary ratio of cortisol to cortisone metabolites in salt-sensitive than salt-resistant subjects after ingestion of 500 mg/d glycyrrhetic acid for one week (Ferrari et al. 2001). Sigurjonsdottir et al. (2003) demonstrated differences in liquorice-induced changes between the genders as the harmful effects were more pronounced in women than in men, while a subsequent study indicated more marked decrease in serum aldosterone concentration in men compared to women (Sigurjonsdottir et al. 2006a). In addition, the marginal responses of serum androgens after liquorice ingestion were different between the genders (Sigurjonsdottir et al. 2006b). Also, genetic factors like polymorphism of 11 β HSD2 may associate with the susceptibility to liquorice (Armanini et al. 2003). However, in a study with subjects with known liquorice-induced hypertension, no mutations in the 11 β HSD2 gene were found, while a contribution of variants of the epithelial sodium channel subunits regulating renal sodium reabsorption was suggested as an alternative explanation (Miettinen et al. 2010).

2.6.2.4 Other effects of liquorice or liquorice-derived compounds

In addition to hypertension, hypokalaemia-related disorders are important side effects of liquorice intake (Omar et al. 2012; Hosseinzadeh and Nassiri-Asl 2015; Nazari et al. 2017). Case reports of liquorice-induced cardiac arrhythmias demonstrated hypokalaemia and subsequent long QT syndrome and ventricular tachycardia of torsades de pointes that may result in cardiac arrest (Eriksson et al. 1999; Crean et al. 2009). Another reported consequence of liquorice-induced hypokalemia is rhabdomyolysis (Shah et al. 2012), that can even be followed by acute kidney injury (Kasap et al. 2010). During pregnancy, glycyrrhizin intake of 500 mg/week or more may shorten the duration of gestation (Strandberg et al. 2001) and

lead to preterm delivery (Strandberg et al. 2002) when compared to zero-low (0-249 mg/week) or moderate (250-499 mg/week) consumption. In addition, when the above-mentioned high intake was compared to zero-low intake of glycyrrhizin, maternal liquorice ingestion during pregnancy was associated with cognitive dysfunction, externalizing symptoms, and attention problems (Räikkönen et al. 2009), and changes in diurnal and stress-induced hypothalamic-pituitary-adrenal axis function (Räikkönen et al. 2010) in the offspring at the age of 8 years in a dose-dependent manner. In addition, liquorice and its bioactive compounds might induce drug-interactions via changes in the activity of the metabolizing enzymes like CYP 450 isoenzymes (Omar et al. 2012; Nazari et al. 2017).

Many pharmacological effects have been suggested for *Glycyrrhiza glabra* due to its bioactive compounds including antioxidative, anti-inflammatory, antiviral, antimicrobial, anticarcinogenic, immunomodulatory, gastroprotective, hepatoprotective and cardioprotective effects (Isbrucker and Burdock 2006; Hosseinzadeh and Nassiri-Asl 2015; Nazari et al. 2017). The liquorice-derivative carbenoxolone has been used for the treatment of gastric and duodenal ulcer (Nagy 1978; Bianchi Porro et al. 1985). However, due to its side-effects and availability of more effective medicines including proton pump inhibitors, the use of carbenoxolone is rare (Hammer and Stewart 2006). In Japan, an intravenous drug containing glycyrrhizin, Stronger Neo-Minophagen C®, has been used for the treatment of liver function abnormalities in chronic liver disease (Fiore et al. 2008). Although liquorice derivatives may possess therapeutic effects in some clinical conditions, regular liquorice intake cannot be recommended as the adverse effects overcome the benefits (Omar et al. 2012).

2.7 Non-invasive assessment of haemodynamics and cardiac autonomic tone

2.7.1 Head-up tilt table test

Cardiovascular reactivity can be measured in a laboratory by tilt table testing, as the change from supine to upright posture results in haemodynamic changes and compensatory responses to maintain the normal BP level (Saal et al. 2016). Due to gravity, blood is transferred from the thorax to the lower limbs, which decreases venous return and CO. The subsequent reduction in BP triggers the baroreflex

resulting in enhanced sympathetic activity and increased HR and SVR (Saal et al. 2016). Tilt table testing has been used in the diagnosis of syncope with varying protocols (Forleo et al. 2013). The haemodynamics can be measured for instance by the use of whole body impedance cardiography (ICG_{WB}) method and applanation tonometry (Tahvanainen et al. 2009a), and the head-up tilt table test can also be performed with pharmacological stimuli (Tahvanainen et al. 2012).

2.7.2 Measurement of cardiac output by whole body impedance cardiography

CO can be measured non-invasively by the ICG_{WB} method. To determine the whole body impedance, an alternating current with frequency of 30 kHz is applied to the pair of current electrodes placed proximally on the wrists and ankles, while the voltage is measured from other electrodes located about 5 cm above the current electrodes (Kööbi et al. 1997b). As the electrical conductance of blood is good, the applied alternating current is passed through the main arterial tree and the changes in the whole body impedance signal are related to the pulsatile blood flow during cardiac cycles (Kööbi et al. 1997b; Kööbi 1999; Cotter et al. 2004). The evaluation of SV by the ICG_{WB} method is based on the detected changes in the whole body impedance signal and usage of a mathematical algorithm (Kööbi et al. 1997a; Kööbi et al. 1997b). Furthermore, HR is measured by the simultaneous recording of electrocardiogram (ECG) with two electrodes placed on the thorax area. Subsequently, CO is calculated based on SV and HR.

The ICG_{WB} method can also be utilised for the determination of aortic-to-popliteal PWV. For this purpose, an active electrode is placed on the lateral side of the knee with a reference electrode about 20 cm below on the calf to record the impedance from a popliteal artery (Kööbi et al. 2003; Koivistoinen et al. 2011). Following the systolic flow ejection the whole-body impedance decreases, which can be measured with the voltage electrodes on the wrists and ankles. The PWV can be calculated from the time difference between the feet of whole body impedance signal and the popliteal artery signal, and the distance measured from the sternal angle to the knee joint level along the body surface (Kööbi et al. 2003; Koivistoinen et al. 2011). With the ICG_{WB} method, the measured CO and PWV values are in a good agreement with the thermodilution method (Kööbi et al. 1997a; Kööbi et al. 1997b) and Doppler ultrasound method (Kööbi et al. 2003), respectively. The placement of

electrodes for the measurement of whole body impedance and aortic-to-popliteal PWV by the ICG_{WB} is depicted in Figure 5.

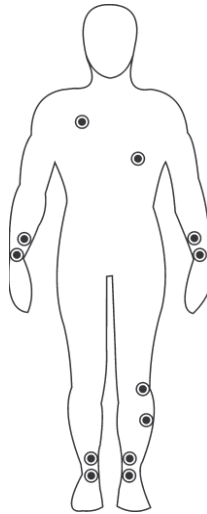


Figure 5. Placement of electrodes in whole body impedance cardiography (on the wrists, ankles, and thorax area) with electrodes on the lateral side of the knee joint and the calf for the measurement of pulse wave velocity. (Adapted from Kööbi et al. 2003 and Koivistoinen et al. 2011).

2.7.3 Central pulse wave analysis by arterial applanation tonometry

Arterial applanation tonometry is a non-invasive method for the estimation of central pressure waveform which can be recorded directly from the carotid artery, or indirectly from the radial artery with a pencil like probe (Laurent et al. 2006). With the SphygmoCor[®] device, radial artery waveform can be transferred into a central one by using a validated generalised transfer function (Karamanoglu et al. 1993; Chen et al. 1997). Thus, the pulse wave analysis from the radial artery produces data about aortic systolic and diastolic BP, and aortic PP and AIx as indicators of central wave reflection. In radial artery tonometry, the recorded radial pressure waveform is calibrated by brachial BP measurements assuming that the radial and brachial pressures are corresponding. The amplitude of arterial pressure pulse is increased towards peripheral circulation, and the aortic to brachial, and brachial to radial systolic BP amplification might vary between individuals (Picone et al. 2018). Recently, four distinct phenotypes have been suggested based on the BP

transmission from the central to peripheral arteries (Picone et al. 2018). Although the accuracy of the transfer function has been criticised, the utilisation of brachial BP measurements for calibration of radial pressure wave is a potential source of error for the method (O'Rourke et al. 2001; Verbeke et al. 2005). However, radial applanation tonometry is considered as a common, non-invasive, and repeatable method for the evaluation of central pressures (O'Rourke et al. 2001; Laurent et al. 2006).

2.7.4 Heart rate variability

Cardiac autonomic tone can be estimated by analysing HR variability (HRV) from either instant HR or RR intervals (that is the time between consecutive heart beats) of the ECG recording, although usually the HRV parameters are calculated from the latter (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996; Xhyheri et al. 2012). The HRV variables can be divided into time domain, frequency domain, and non-linear methods (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). The frequency domain method is recommended for short-term recordings, and components including high frequency (HF) power (frequency range 0.15 to 0.4 Hertz) and low frequency (LF) power (frequency range 0.04 to 0.15 Hertz) can be calculated utilising the power spectral density analysis with either parametric (autoregressive model) or non-parametric (Fast Fourier Transformation) method. The HF power is a marker of cardiac parasympathetic activity, while LF power predominantly reflects sympathetic activity, although it also influenced by vagal contributions (Xhyheri et al. 2012). The ratio of LF to HF power has been proposed to evaluate the balance of cardiac autonomic tone (Xhyheri et al. 2012). Of note, HRV is associated with the average HR as a consequence of both physiological and mathematical interactions (Sacha 2014). The possible mathematical bias of the HRV analysis is due to the nonlinear relationship between RR interval and HR. With slow average HR the variability of RR intervals is higher than with fast average HR, which leads in increasing HRV when HR slows and vice versa (Sacha 2014). However, the HRV parameters can be mathematically modified to decrease the HRV dependence on HR (Sacha 2014), and measurement of HRV is considered as a useful method to estimate cardiac autonomic tone (Stauss 2014).

3 AIMS OF THE STUDY

Since the existing data about the effects of LTPs on BP is not consistent, the aim of the present investigation was to study the effects of Ile-Pro-Pro and Val-Pro-Pro, two compounds with previously suggested RAAS inhibiting properties, on haemodynamics in subjects with the MetS utilising a comprehensive non-invasive measurement protocol of haemodynamics with orthostatic challenge. In addition, the present investigation examined the detailed haemodynamic alterations leading to the elevation of BP after liquorice consumption, a condition that is characterised by the suppression of the RAAS due to the inhibition of the enzyme 11 β HSD2, which results in intracellular accumulation of cortisol.

The specific aims of the study were:

1. To study the long-term effects of intake of fermented milk product containing LTPs and PSe on haemodynamics in subjects with the MetS. Two doses of LTPs (5 and 25 mg/d) were tested, and as parallel lowering of plasma lipids is expected to increase the beneficial cardiovascular effects, the test products also included 2 g/d of PSe (**I**).
2. To investigate the haemodynamic changes underlying the liquorice-induced elevation of BP in normotensive volunteers in the supine position (**II**).
3. To examine the cardiovascular influences of liquorice ingestion during orthostatic challenge in healthy subjects (**III**).
4. To study whether vasodilatation induced by exogenous NO donor (nitroglycerin) or β_2 -adrenoceptor stimulation (salbutamol) is impaired after regular liquorice intake in healthy volunteers, and examine whether the liquorice-induced suppression of 11 β -HSD2 activity associates with the observed haemodynamic alterations (**IV**).

4 SUBJECTS AND METHODS

4.1 Study subjects and design

Study I

One hundred and sixteen individuals with elevated systolic or diastolic BP of ≥ 140 or ≥ 85 mmHg, respectively, and MetS according to the IDF criteria (Alberti et al. 2005) were included in a randomised, double-blind, and placebo controlled study. Subjects with BP- or lipid-lowering medication, secondary hypertension, unstable coronary artery disease, diabetes, malignancy, or milk allergy were excluded. Also, smoking, alcohol overuse, pregnancy, and breast feeding were criteria for exclusion from the study. After a run-in period of 4 weeks, the subjects were randomised to 3 parallel groups to consume fermented milk product containing 1) 5 mg/d LTPs + 2 g/d PSe (LTP5 + PSe), 2) 25 mg/d LTPs + 2 g/d PSe (LTP25 + PSe), or 3) placebo without added LTPs and PSe for 12 weeks. One subject dropped out during the run-in period, and altogether 5 subjects during the intervention period due to personal reasons. In addition, due to unsuccessful haemodynamic recordings and delayed reporting of statin and metformin therapy, altogether 6 more subjects were excluded from the final analysis. The demographic characteristics of the study subjects are presented in Table 2, and regular medications used by the subjects in Table 3.

The volunteers contacted the study nurse based on announcements in a local newspaper. At the screening visit, the suitability of the subjects was checked by a structured interview and laboratory examinations. The medications, dietary supplements and lifestyle habits were documented. During the study, a fermented milk product of 125 ml was incorporated to the subjects' diet twice a day, but otherwise the subjects were instructed to continue their possible medical therapies and usual lifestyle habits including diet and exercise throughout the study. The haemodynamic recordings, laboratory tests, and body weight measurement were performed at weeks 0, 8 and 12. Laboratory tests included sodium, potassium, lipids, aldosterone, and renin activity (weeks 0, 8 and 12), and full blood count, creatinine, glucose, C-reactive protein, uric acid, calcium, and phosphate (weeks 0 and 12). In addition, the subjects measured BP at home twice a week during the intervention.

Table 2. Characteristics of the study subjects in studies I-IV

| Study I | Placebo n=33 | LTP5+PSe n=36 | LTP25+PSe n=35 | |
|--------------------------------------|-----------------------------------|-----------------------------|-------------------------------|-------------------------------------|
| Age (years) | 50.2 ± 7.2 | 49.5 ± 6.9 | 54.3 ± 5.9 | |
| Male/female | 20/13 | 21/15 | 22/13 | |
| Body mass index (kg/m ²) | 30.0 ± 3.7 | 30.5 ± 4.5 | 31.5 ± 3.5 | |
| Office systolic BP (mmHg) | 162 ± 15 | 159 ± 12 | 168 ± 20 | |
| Office diastolic BP (mmHg) | 100 ± 10 | 97 ± 8 | 102 ± 10 | |
| Studies II-IV | Control, n=30 (studies II-III) | Control, n=21 (study IV) | Liquorice, n=20 (study II) | Liquorice, n=22 (studies III-IV) |
| Age (years) | 33.8 ± 8.1 | 47.3 ± 6.2 | 33.5 ± 7.9 | 34.9 ± 9.2 |
| Male/female | 13/17 | 18/3 | 8/12 | 8/14 |
| Body mass index (kg/m ²) | 23.0 ± 2.7 | 28.2 ± 3.3 | 23.3 ± 1.9 | 23.3 ± 1.9 |
| Office systolic BP (mmHg) | 116 ± 14 | 134 ± 9 | 119 ± 8 | 119 ± 8 |
| Office diastolic BP (mmHg) | 72 ± 11 | 85 ± 7 | 70 ± 8 | 70 ± 7 |

LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

Mean values ± standard deviations depicted. BP, blood pressure.

Table 3. Regular medications of the subjects in study I. The number of subjects with each type of pharmacological therapy depicted.

| | Placebo (n=33) | LTP5+PSe (n=36) | LTP25+PSe (n=35) | All (n=104) |
|-----------------------------|-------------------|--------------------|---------------------|----------------|
| Hormone replacement therapy | 1 | 6 | 1 | 8 |
| SSRI | 2 | 2 | 4 | 8 |
| Thyroxine | 2 | 4 | 2 | 8 |
| Corticosteroids, topical | 1 | 2 | 0 | 3 |
| Acetylsalicylic acid | 0 | 0 | 2 | 2 |
| Antiepileptic agents | 0 | 0 | 2 | 2 |
| Benzodiazepines | 0 | 2 | 0 | 2 |
| Tizanidine | 1 | 1 | 0 | 2 |
| SNRI | 0 | 2 | 0 | 2 |
| Alfuzosin | 0 | 1 | 0 | 1 |
| Antimicrobials | 0 | 1 | 0 | 1 |
| Brimonidine eye drops | 1 | 0 | 0 | 1 |
| Dopamine agonists | 0 | 0 | 1 | 1 |
| Flecainide | 1 | 0 | 0 | 1 |
| Glucosamine | 0 | 0 | 1 | 1 |
| Latanoprost eye drops | 1 | 0 | 0 | 1 |
| NSAID | 1 | 0 | 0 | 1 |
| Proton pump inhibitor | 0 | 1 | 0 | 1 |
| Salazosulphapyridine | 1 | 0 | 0 | 1 |
| Salmeterol | 0 | 1 | 0 | 1 |
| Timolol eye drops | 1 | 0 | 0 | 1 |
| Tramadol | 0 | 1 | 0 | 1 |
| Tricyclic antidepressant | 0 | 1 | 0 | 1 |

LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

NSAID=non-steroidal anti-inflammatory agent, SSRI=selective serotonin reuptake inhibitor, SNRI=serotonin-norepinephrine reuptake inhibitor. Topical corticosteroids include nasal and inhaled preparations.

Studies II, III, and IV

The study population in the liquorice group consisted of normotensive, healthy volunteers as depicted in Table 2, and the data of these subjects were included in the analyses of studies II-IV. The results of the liquorice group were compared with an age-matched group of 30 people in studies II and III, while in study IV, the liquorice data was analysed in parallel with another control group of 21 people (Table 2). The studies II and III examined the haemodynamic alterations after 2 weeks of daily liquorice exposure at rest and during orthostatic challenge, respectively. The study IV investigated the effects of liquorice consumption on the haemodynamic responses induced by sublingual nitroglycerin and inhaled salbutamol during orthostatic challenge. Since repeated haemodynamic recordings with administration of these drugs were not performed in the control subjects of the studies II and III, another reference group was required for study IV.

The recruitment of the subjects in the studies II-IV was performed via announcements distributed within the University of Tampere, Tampere University Hospital, occupational health care units, and Varala Sports Institute, and also in a local newspaper. The study design was open-label. The controls had participated in an on-going investigation on haemodynamics (the DYNAMIC study), and did not know of forming the control group for the liquorice intervention. Exclusion criteria for the liquorice study were elevated systolic/diastolic office BP of $\geq 140/90$ mmHg, any cardiovascular disease with regular drug therapy, pregnancy, and previous high intake of liquorice of >300 g per week. The control subjects were without BP-lowering medication, heart disease, diabetes, and cerebrovascular and peripheral arterial disease. The regular medications and smoking status of the subjects in studies II-IV are presented in Table 4.

During the 2-week liquorice intervention, the subjects' daily dose of glycyrrhizin ranged 290-370 mg from the commercial liquorice products used in the study. Before the initiation of the liquorice intervention, the subjects were instructed not to consume products containing glycyrrhizin for 3 weeks. The control subjects of the studies II-IV were instructed to maintain their habitual diet. The suitability of all the subjects for studies II-IV were ensured with a medical examination and an interview of medical and family history, and lifestyle habits by a physician. The lifestyle questionnaire also included an estimation for liquorice consumption and according to this, the habitual liquorice intake frequency was less than weekly among the control subjects of studies II and III, while in the study IV the reported liquorice

consumption frequency was weekly among 4 control subjects and less than weekly among 17 control subjects.

The haemodynamic recordings were performed at the beginning and end of the 2-week liquorice intervention, and after 1-3 weeks follow-up (controls in the studies II-IV). However, in the study IV, the duration of follow-up was 10 months in 5 control subjects. In study III, autonomic tone was evaluated by spectral analysis of heart rate variability at the beginning and end of the study. Laboratory tests were drawn at baseline. In the liquorice group, plasma samples for determination of potassium, sodium, creatinine, aldosterone, and renin activity, and 24-h urine collection for determination of cortisone and cortisol metabolites were performed at weeks 0 and 2. The haemodynamic recording data with nitroglycerin and salbutamol (IV) was absent in 1 male and 1 female subject in the liquorice group at the final visit due to nitroglycerin-induced headache after baseline recordings and technical problems, respectively.

Table 4. Regular medications and smoking status of the subjects in studies II-IV. Number of subjects depicted.

| | Studies II and III Control n=30 | Study IV Control n=21 | Studies II-IV Liquorice n=20-22 |
|------------------------------------------|------------------------------------|--------------------------|------------------------------------|
| Medications | | | |
| Intramuscular vitamin B12 replacement | 0 | 1 | 0 |
| Combination of budesonide and formoterol | 0 | 0 | 1 |
| Female hormones* | 8 | 1 | 6 |
| Proton pump inhibitor | 0 | 2 | 0 |
| SSRI | 0 | 2 | 1 |
| Statin | 0 | 3 | 0 |
| Thyroxine | 0 | 1 | 0 |
| Smoking status | | | |
| Present | 3 | 0 | 4 |
| Previous | 4 | 9 | 1 |
| Never | 23 | 12 | 17 |

*Peroral medications and intrauterine devices
SSRI, selective serotonin reuptake inhibitor

4.2 Haemodynamic measurement protocol

The haemodynamic recordings were performed at Tampere University Hospital, Department of Clinical Physiology by trained research nurses under temperature-controlled conditions (Tahvanainen et al. 2009a; Tahvanainen et al. 2012; Koskela et al. 2013). The subjects were asked to refrain from caffeine-containing products,

smoking, and heavy meal (for ≥ 4 hours), and alcohol (for ≥ 24 hours) prior to the recordings. At the beginning of the haemodynamic recording protocol the subjects were resting supine on the tilt-table, and the electrodes for ICG_{WB} were set on the body surface and an automated tonometric sensor for pulse wave analysis on the left radial pulsation with a wrist band. In addition, a cuff was placed to the right upper arm in order to calibrate the radial BP signal approximately every 2.5 min. By using a bracket, the extended left arm was held at the level of the heart during both supine and upright measurements (Tahvanainen et al. 2009a; Tahvanainen et al. 2012; Koskela et al. 2013).

The haemodynamic data was captured continuously during consecutive 5-min periods (Tahvanainen et al. 2009a). After the first 5 min recordings in the supine position, a passive head-up tilt to 60 degrees was performed for 5 min. Following this, the tilt-table was restored to supine position for 5 min. In study II, the haemodynamic measurements were performed during the first 5 min at rest, while studies I, III, and IV included the passive head-up tilt (15 min). In study IV, the recording protocol was repeated after administration of 0.25 mg sublingual nitroglycerin resoriblet (Nitro resoriblet; Orion Pharma, Espoo, Finland) (Tahvanainen et al. 2012). In addition, the haemodynamics were recorded before and after administration of 400 μ g salbutamol inhalation (Ventoline; GlaxoSmithKline, Uxbridge, Middlesex, UK) after a one hour pause following the nitroglycerin-stimulated measurements (IV). A spacer device was applied to deliver the salbutamol inhalation (Volumatic; Allen & Hanbury's, Uxbridge, Middlesex, UK) after the subject had become familiar with the inhalation technique. In case of presyncopal symptoms and falling BP in response to orthostatic challenge after sublingual nitroglycerin, the research nurse aborted the head-up tilt before the intended 5 min were carried out (Tahvanainen et al. 2011). Previous studies have demonstrated a good reproducibility and repeatability for the haemodynamic measurement protocol (Tahvanainen et al. 2009a; Tahvanainen et al. 2012).

4.2.1 Pulse wave analysis

A tonometric sensor was used to determine continuous pulse wave form and radial BP from the left radial pulsation (Colin BP-508T, Colin Medical Instruments Corp., San Antonio, Texas, USA). The SphygmoCor[®] pulse wave monitoring system (SphygmoCor PWMx, Atcor Medical, Australia) was applied to estimate continuous aortic BP from the radial signal with validated generalized transfer function (Chen et

al. 1997). The AIx and PP were determined from the aortic pulse wave form. The central forward wave amplitude (FWA) was defined as the difference between pressure at the waveform foot and pressure at the first systolic inflection point of the aortic pressure waveform (Mitchell et al. 2004; Kaess et al. 2012).

4.2.2 Whole-body impedance cardiography

Beat-to-beat HR, SV, cardiac index (CI, equal to CO per body surface area, l/min/m²), and ECW volume were determined by measuring the changes in body electrical impedance during a cardiac cycle with a ICG_{WB} device (CircMon®, JR Medical Ltd., Tallinn, Estonia) (Kööbi et al. 1997a; Kööbi et al. 1997b; Kööbi et al. 2003). The radial BP signal and CI were used to calculate the systemic vascular resistance index (SVRI, equal to SVR per body surface area, dyn*s/cm⁵/m²). The PWV was determined by measuring the pulse transit time from the aortic arch to the popliteal artery at knee joint level through the time difference between the feet of whole body impedance signal and the popliteal artery signal, and the distance between the electrodes (Kööbi et al. 1997a; Kööbi et al. 2003). The PWV values obtained by the CircMon® software are in agreement with the values measured with Doppler ultrasound (Kööbi et al. 2003) and SphygmoCor® applanation tonometry (Wilenius et al. 2016). In addition, the SV and CO determined with the whole-body impedance cardiography correlate well with measurements utilising 3-dimensional echocardiography and the thermodilution method, respectively (Kööbi et al. 1997a; Koskela et al. 2013).

4.2.3 Frequency domain analysis of heart rate variability

In study III, cardiac autonomic tone was assessed utilising HRV analysis from the ECG recorded by the CircMon® device (sampling rate 200 Hz), and data were analysed using Matlab software (MathWorks Inc., Natick, Massachusetts, USA) (Kangas et al. 2016; Tahvanainen et al. 2016). Normal R-R intervals were identified, and if the interval deviated over 20 % from the previous values beat was regarded as ectopic. The cubic spline interpolation method was used to process the artefacts (Peltola 2012). The Fast Fourier Transformation method was used to calculate the frequency domain variables: i) power in LF range (0.04–0.15 Hz), ii) power in HF range (0.15–0.40 Hz), and iii) LF/HF ratio (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996).

4.3 Brachial office and home blood pressure measurements

In study I, office BP was measured using an automated sphygmomanometer (Omron M4; Omron Matsusaka Limited) in the sitting position after 5 min rest at the screening visit and after 12 weeks of intervention (Williams et al. 2018). The measurement was made in duplicate, and if the BP values differed more than 5 mmHg, another measurement was performed. In case of several measurements, the BP values in which the difference was less than 5 mmHg were included in the statistical analyses. Furthermore, study I comprised home BP measurements twice a week (on one week day and one weekend day) during the intervention. The subjects were carefully instructed to perform the measurements in duplicate with automated sphygmomanometer (Omron M4) about 1 hour after waking in the morning. In studies II-IV, office brachial BP was determined manually with a standard aneroid sphygmomanometer (Heine Gamma G7, HEINE Optotechnik, Herrsching, Germany) in the sitting position during the medical examination, while BP was also measured by the research nurses in the supine position before the laboratory recordings of pulse wave analysis and ICG_{WB}. In these measurements, the phase I and V Korotkoff sounds were used to identify systolic and diastolic BP, respectively.

4.4 Laboratory measurements

Height, waist, and hip circumference were recorded to the closest 0.5 cm at screening, and body weight was measured with a digital scale at every visit while the subjects were wearing light clothing. Standard 12-lead ECGs were obtained with MAC5000 (GE Healthcare, Chalfont St. Giles, UK) at baseline. Venous blood samples were collected after about 12 hours of fasting for the determination of blood count (ADVIA 120 or 2120, Bayer Health Care, Tarrytown, NY, USA), and plasma sodium, potassium, creatinine, glucose, triglyceride, total cholesterol, HDL-cholesterol, and LDL-cholesterol (Cobas Integra 700/800, F. Hoffmann-LaRoche Ltd, Basel, Switzerland), and plasma insulin concentration (Cobas e411 analyser, RocheDiagnostics). The estimated glomerular filtration rate was calculated with the RULE formula (Rule et al. 2004), since plasma creatinine levels were within the normal range among the subjects. Plasma renin activity was determined utilising GammaCoat Plasma Renin Activity assay (Diasorin) and aldosterone concentration by active aldosterone RIA (Diagnostics Systems Laboratories, Beckman Coulter). Urinary THE and allo-THF+THF concentrations were analysed from 24-h urine

collection by liquid chromatography tandem mass spectrometry (Turpeinen et al. 2006).

4.5 Study products

4.5.1 Study I

The nutrient content of the fermented milk products used during the run-in and intervention period are presented in Table 5. The run-in product was different from the test and placebo products. It did not contain LTPs or PSe, and the mineral (calcium, potassium, and magnesium) content was smaller than in the test products. The LTPs were added to the test products as peptide powder manufactured from *Lactobacillus helveticus* Lc 1936 fermented milk using nanofiltration and drainage. Free plant sterols were esterified with fatty acids from vegetable oil (Cognis Corporation, Illertissen, Germany), and produced PSe was included in the test products so that the content of β -sitosterol was max 80 %, campesterol max 40 %, stigmasterol max 30 %, β -sitostanol max 15 % and campestanol max 5 %, while other sterols and stanols were present at <5%. The daily dosage of LTPs, PSe, and minerals are shown in Table 5. No bioactive LTPs or PSe were present in the placebo product. All the fermented milk products were manufactured by Valio Ltd., Helsinki, Finland.

4.5.2 Studies II-IV

The glycyrrhizin content of the commercial liquorice products (Halva liquoriceTM and Kouvola liquoriceTM) used in studies II-IV were determined in 1993 (Blomberg and Hallikainen 1993). After this, the preparation processes have not changed, thus the daily amount of HalvaTM and KouvolaTM liquorice was calculated based on the report of Blomberg and Hallikainen (1993). Depending on the glycyrrhizin content of the liquorice product, the amount of ingested liquorice was 120 g of Halva liquoriceTM, or 300 g of Kouvola liquoriceTM, or combination of these two products, targeting for glycyrrhizin dose of 290-370 mg/d. The average amount of carbohydrates from the liquorice products was 150 g/d corresponding an energy intake of 600 kcal/d. The liquorice products were manufactured by Kouvola Lakritsi Oy and Oy Halva Ab.

Table 5. Nutrient content of the fermented milk products per daily dose of 250 ml (study I)

| | Run-in product | Placebo product | LTP5+Pse product | LTP25+Pse product |
|--------------------------------|----------------|-----------------|------------------|-------------------|
| Energy (kcal) | 181 | 169 | 167 | 167 |
| Fat (g) | 0.25 | 1 | 3 | 3 |
| Protein (g) | 9.7 | 8 | 10 | 10 |
| Carbohydrates (g) | 35 | 32 | 25 | 25 |
| Calcium (mg) | 300 | 250 | 380 | 380 |
| Potassium (mg) | 375 | 375 | 660 | 660 |
| Magnesium (mg) | 27.5 | 27.5 | 41 | 41 |
| Ile-Pro-Pro + Val-Pro-Pro (mg) | - | - | 5 | 25 |
| Plant sterol esters (g) | - | - | 2 | 2 |

LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

4.6 Statistical analyses

The variable values are expressed as means with standard deviation (SD), standard error of the mean, or 95 % confidence interval. Mean beat-to-beat values of each minute of the haemodynamic recording protocol were calculated for the statistical analyses. In studies I, III, and IV, the mean supine and upright haemodynamic values were calculated from the minutes 3-5, when the signal was most stable, while in study II the mean values from the whole 1-5 min period were used in the analyses. Also, in study III the results of LF and HF power were analysed using the mean supine and upright values from the whole 5 min period. In study I, home and office BP values are expressed as the mean of two measurements. In the analyses of home BP, the recordings from the last week of each period were utilised (**I**). In study IV, if the nitroglycerin-stimulated head-up tilt had to be aborted due to presyncopal symptoms, the absent values were replaced by the preceding values, providing that at least 2 min of 5 min upright recordings were captured. The normal distribution of the variables was tested with the Shapiro-Wilk test and histograms, and the homogeneity of variances with the Levene's test. The LF and HF power were divided by the average RR interval squared to diminish the HRV dependence on HR (Sacha 2014), and converted to natural logarithm to normalise the distributions prior to statistical analyses (**III**). In study I, univariate analysis of variance (ANOVA) was utilised to test baseline data between the groups with age as covariate, and changes between the groups with baseline value as covariate. In studies II-IV, independent samples t-test or Mann-Whitney test was used to test baseline values and changes between the groups. In within-group comparisons, paired samples t-test or Wilcoxon signed ranks test was applied.

ANOVA for repeated measurements was used to test the interaction of measurement time and group, and differences between the groups in haemodynamic variables during rest and orthostatic challenge. To study the effect of intervention on haemodynamic variables during the recording protocol, the changes from week 0 to week 12 (**I**) and from week 0 to week 2 (**III**) were calculated for each minute and compared between the groups with ANOVA for repeated measurements. In study IV, due to baseline differences between the liquorice and control groups (see section 5.2.1 below), the changes during the study were analysed separately within the groups. In these analyses, ANOVA for repeated measurements was applied to study differences in haemodynamic variables between the study visits during supine and upright position. In addition, ANOVA for repeated measurements was used to compare differences between measurements and groups in home BP measurements (**I**) and HRV (**III**). Adjustment for age was made in study I. In post hoc comparisons Bonferroni correction was applied.

For non-continuous variables Chi-square test was applied, and Spearman's correlations (r_s) were calculated, as appropriate. The statistical analyses were performed utilising IBM SPSS Statistics Version 17 (SPSS Inc., Chicago, Illinois, USA) or Version 24 (IBM Corporation, Armonk, NY, USA), and $P < 0.05$ was considered statistically significant.

4.7 Ethics

All of the studies I-IV were approved by the Ethics Committee of the Tampere University Hospital (study code R08012 for study I and R07053M for studies II-IV), conformed to the principles stipulated in the Declaration of Helsinki, and are registered in the database of clinical trials (ClinicalTrials.gov, ID: NCT01742702). The studies I-IV are part of an on-going investigation on haemodynamics (DYNAMIC-study; EudraCT-number 2006-002065-39) approved by the Ethics Committee of Tampere University Hospital and by the Finnish Medicines Agency (code numbers R06086M). All participants gave a written informed consent.

5 RESULTS

5.1 Fermented milk products containing lactotripeptides and plant sterol esters in subjects with the metabolic syndrome (Study I)

5.1.1 Study subjects and adherence to study product consumption

The subjects in the LTP25+PSe group were 4 to 5 years older compared to the LTP5+PSe and placebo group (Table 6). In addition, there was a numerical but not statistically significant tendency towards higher waist circumference of female subjects in the LTP25+PSe group than the other two groups at baseline. However, no significant differences were detected in sex distribution and laboratory tests (Table 6), or weight (Table 7). Furthermore, weight remained stable during the 12-week intervention among the subjects (Table 7), as well as plasma glucose concentration since the mean changes (95 % CI) were 0.03 (-0.12; 0.17), 0.06 (-0.10; 0.21), and 0.03 (-0.10; 0.17) mmol/l in the placebo, LTP5+PSe, and LTP25+PSe group, respectively ($p=0.826$ between the groups). No significant changes were detected in plasma potassium and aldosterone concentrations, or renin activity during the intervention between the study groups (Table 7). The intake of study products was less than 80 % in one subject in the LTP5+PSe group, but otherwise the reported compliance was excellent with mean (SD) values of 99 (3) %, 98 (4) % and 99 (3) % in the placebo, LTP5+PSe, and LTP25+PSe groups, respectively.

5.1.2 Haemodynamic recording protocol in the laboratory

In the baseline measurements, the study groups showed significant differences in the regulation of radial systolic ($p=0.002$) and diastolic ($p=0.004$) BP, and SVRI ($p=0.001$), but not CI ($p=0.276$) during orthostatic challenge (Figure 6 and 7). As the clearest baseline difference in functional haemodynamic regulation, the LTP5+PSe group had lower diastolic BP and SVRI than the placebo group at rest, although the levels of upright values were similar.

The haemodynamic measurements performed after the 12-week intervention are shown in Figure 6 and 7. To study the effects of LTP+PSe products on haemodynamic variables, the changes from week 0 to week 12 during the haemodynamic recording protocol were calculated and compared between the three groups. ANOVA for repeated measurements (age as covariate) indicated no significant time*group interactions or between group differences in radial systolic and diastolic BP, SVRI or CI. Also, the measurements at week 8 indicated no significant effects of LTP+PSe products on haemodynamics (data not shown).

Table 6. Demographic and clinical characteristics of the subjects at baseline in study I

| | Placebo (n=33) | LTP5+PSe (n=36) | LTP25+PSe (n=35) | P ANOVA* |
|-----------------------------------------|-------------------|--------------------|---------------------|----------|
| Age (years) | 50.2 ± 7.2 | 49.5 ± 6.9 | 54.3 ± 5.9†‡ | 0.007 |
| Male/Female | 20/13 | 21/15 | 22/13 | 0.927 |
| Waist circumference (cm) | | | | |
| Male | 106 ± 9 | 109 ± 9 | 109 ± 7 | 0.291 |
| Female | 95 ± 8 | 97 ± 11 | 104 ± 10 | 0.071 |
| Haematocrit (%) | 0.43 ± 0.04 | 0.43 ± 0.03 | 0.42 ± 0.03 | 0.993 |
| Fasting plasma | | | | |
| Glucose (mmol/l) | 5.7 ± 0.5 | 5.8 ± 0.7 | 5.9 ± 0.6 | 0.528 |
| Insulin (mU/l) | 9 ± 3 | 10 ± 7 | 8 ± 4 | 0.406 |
| Total cholesterol (mmol/l) | 5.7 ± 0.8 | 5.6 ± 1.0 | 5.7 ± 0.6 | 0.903 |
| LDL-cholesterol (mmol/l) | 3.5 ± 0.7 | 3.5 ± 0.8 | 3.5 ± 0.7 | 0.875 |
| HDL-cholesterol (mmol/l) | 1.34 ± 0.31 | 1.32 ± 0.39 | 1.42 ± 0.34 | 0.944 |
| Triglycerides (mmol/l) | 1.77 ± 0.86 | 2.05 ± 1.14 | 1.80 ± 0.88 | 0.535 |
| eGFR§ (ml/min per 1.73 m ²) | 111 ± 14 | 113 ± 14 | 106 ± 12 | 0.713 |

LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

Mean values ± standard deviations depicted.

*Age as covariate.

†Significantly different from LTP5+Pse (p=0.009†) and placebo (p=0.038‡).

°Chi-square test

§eGFR, estimated glomerular filtration rate calculated with the RULE formula (Rule et al. 2004).

5.1.3 Blood pressure measurements at home

The mean values of home BP measurements during the 12-week intervention are shown in Table 8 and the changes after 12 weeks in Table 9. Systolic and diastolic BP was numerically decreased in all study groups during the study. However, no antihypertensive effects were detected for the LTP5+PSe and LTP25+PSe treatments as the time*group interactions were not statistically significant (Table 8). The level of home diastolic BP was lower in the LTP5+PSe than LTP25+PSe group (p=0.009, Table 8).

Table 7. Changes in body weight, plasma aldosterone concentration and renin activity, and plasma potassium concentration after the 12-week intervention (study I)

| | Placebo (n=33) | | LTP5+PSe (n=36) | | LTP25+PSe (n=35) | | P* |
|-------------------------|----------------|-----------------|-----------------|-----------------|------------------|-----------------|-------|
| | Mean | SD (95 % CI) | Mean | SD (95 % CI) | Mean | SD (95 % CI) | |
| Weight (kg) | | | | | | | |
| Baseline | 89.8 | 14.2 | 92.1 | 17.6 | 93.7 | 12.3 | 0.070 |
| Change | +0.2 | (-0.4; 0.8) | -0.1 | (-0.7; 0.5) | +0.5 | (-0.3; 1.2) | 0.307 |
| Aldosterone (pmol/l) | | | | | | | |
| Baseline | 499 | 192 | 450 | 206 | 483 | 223 | 0.532 |
| Change | +11 | (-71; 94) | +36 | (-62; 134) | +18 | (-54; 91) | 0.912 |
| Renin activity (µg/l/h) | | | | | | | |
| Baseline | 0.90 | 0.74 | 0.58 | 0.48 | 0.69 | 0.68 | 0.111 |
| Change | +0.14 | (-0.06; 0.33) | +0.22 | (-0.04; 0.48) | +0.10 | (-0.09; 0.29) | 0.953 |
| Potassium (mmol/l) | | | | | | | |
| Baseline | 4.0 | 0.3 | 4.0 | 0.3 | 4.1 | 0.2 | 0.749 |
| Change | -0.1 | (-0.2; 0.0) | -0.1 | (-0.2; -0.0) | -0.2 | (-0.2; -0.1) | 0.597 |

LTP5+PSe, 5 mg/d lactotriptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotriptides and 2 g/d plant sterol esters.

Mean values and 95 % confidence intervals depicted.

*Univariate ANOVA with age as covariate (baseline), and age and baseline value as covariates (change in 12 weeks).

Table 8. Systolic and diastolic blood pressure (BP) measured at home during study I

| | Placebo (n=32) | LTP5+PSe (n=36) | LTP25+PSe (n=35) | ANOVA for repeated measures P* | | |
|----------------------------|----------------|-----------------|------------------|--------------------------------|--------------------------|-------------------------|
| | | | | Time*group interaction | Between-group difference | Within-group difference |
| Systolic BP (mmHg) | | | | | | |
| Baseline | 139 ± 14 | 139 ± 11 | 147 ± 17 | | | |
| 8 weeks | 138 ± 14 | 137 ± 13 | 144 ± 17 | | | |
| 12 weeks | 137 ± 15 | 138 ± 13 | 145 ± 17 | 0.746 | 0.055 | 0.170 |
| Diastolic BP (mmHg) | | | | | | |
| Baseline | 87 ± 9 | 85 ± 7 | 91 ± 10 | | | |
| 8 weeks | 85 ± 8 | 84 ± 8 | 89 ± 9 | | | |
| 12 weeks | 86 ± 9 | 84 ± 8 | 89 ± 9 | 0.726 | 0.011† | 0.299 |

LTP5+PSe, 5 mg/d lactotriptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotriptides and 2 g/d plant sterol esters.

Mean values ± standard deviations depicted.

*Age as covariate; †LTP25+PSe group was different from LTP5+PSe group (p=0.009), Bonferroni corrections applied in post-hoc comparisons.

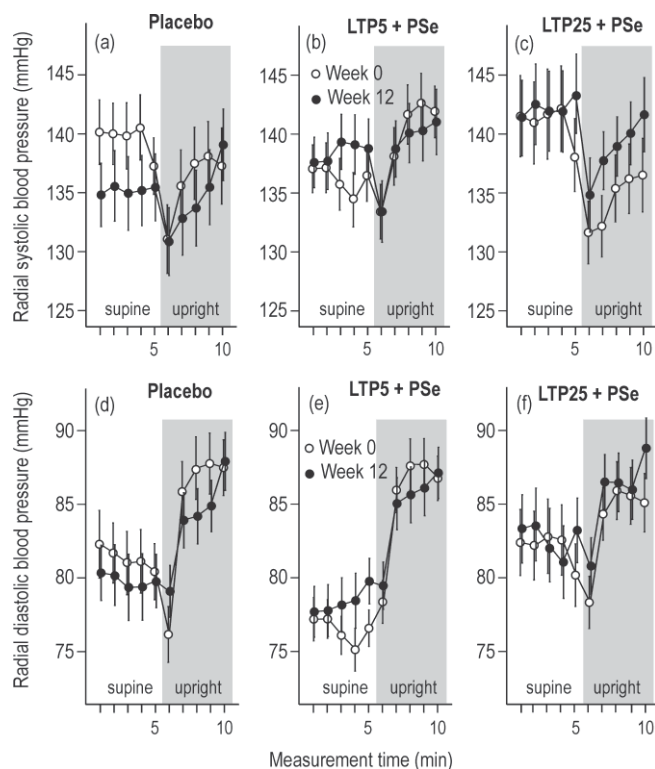


Figure 6. Radial systolic blood pressure (a, b, and c) and diastolic blood pressure (d, e, and f) during the 10-min haemodynamic recording protocol at baseline and after 12 study weeks (study I). Mean and standard error of the mean depicted. Significant time*group interaction was detected in radial systolic blood pressure ($p=0.002$) and diastolic blood pressure ($p=0.004$) at baseline. LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

5.1.4 Plasma lipids and lipoproteins

After 12 weeks of study product consumption, no significant changes were observed in plasma total cholesterol, HDL-cholesterol and triglyceride concentrations in the groups (Table 9). LDL-cholesterol was slightly (numerically) decreased after LTP5+PSe and LTP25+PSe treatments, but the changes between the study groups did not reach statistical significance ($p=0.066$). However, after combining the data of the two LTP-groups with daily dose of 2 g PSe, the test product treatment induced significant reduction in LDL-cholesterol compared to placebo ($p=0.024$).

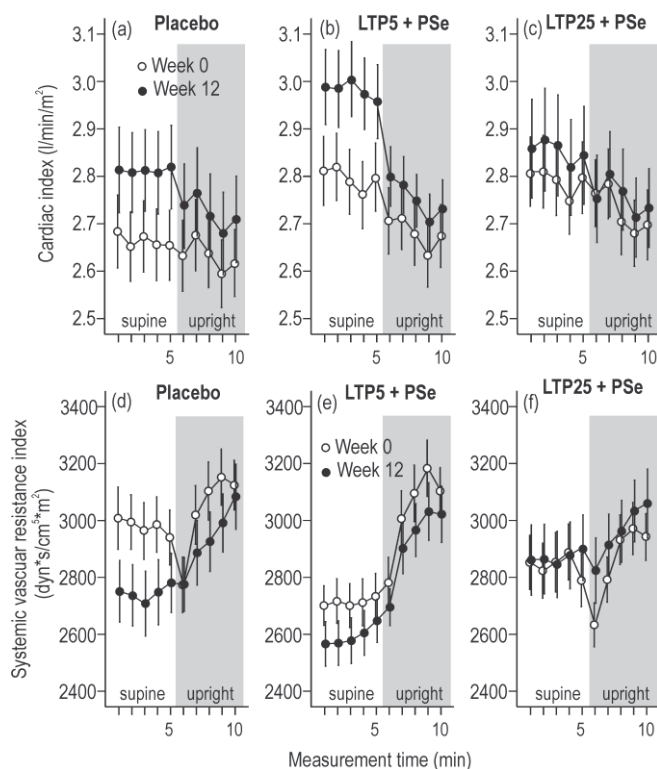


Figure 7. Cardiac index (a, b, and c) and systemic vascular resistance index (d, e, and f) during the 10-min haemodynamic recording protocol at baseline and after 12 study weeks (study I). Mean and standard error of the mean depicted. Significant time*group interaction was detected in systemic vascular resistance index ($p=0.001$) at baseline. LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

Table 9. Changes in home blood pressure (BP) and plasma lipids after the 12-week intervention (study I)

| | Placebo (n=32-33*) | | LTP5+PSe (n=36) | | LTP25+PSe (n=35) | | P† |
|-------------------------------|--------------------|--------------|-----------------|-------------|------------------|-------------|-------|
| | Mean | 95 % CI | Mean | 95 % CI | Mean | 95 % CI | |
| Home BP (mmHg) | | | | | | | |
| Systolic BP | -2.4 | -5.3; 0.5 | -1.4 | -3.9; 1.2 | -1.1 | -4.7; 2.5 | 0.274 |
| Diastolic BP | -1.3 | -3.1; 0.4 | -1.3 | -3.0; 0.4 | -1.0 | -3.1, 1.1 | 0.256 |
| Plasma lipids (mmol/l) | | | | | | | |
| Total cholesterol | +0.1 | -0.1; 0.2 | -0.0 | -0.3; 0.2 | -0.1 | -0.3; 0.1 | 0.465 |
| LDL-cholesterol | +0.1 | -0.1; 0.3 | -0.1 | -0.3; 0.1 | -0.1 | -0.3; 0.0 | 0.066 |
| HDL-cholesterol | -0.08 | -0.15; -0.02 | -0.06 | -0.12; 0.00 | -0.05 | -0.12; 0.01 | 0.716 |
| Triglycerides | +0.11 | -0.20; 0.42 | +0.02 | -0.32; 0.36 | +0.10 | -0.10; 0.29 | 0.742 |

LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters. Mean values and 95 % confidence intervals depicted. *Home BP measurements were not obtained from one subject. †Univariate ANOVA with age and baseline value as covariates.

5.2 Regular liquorice ingestion in healthy volunteers (Studies II-IV)

The P-values in the Figures 8-10 are for the changes in haemodynamic variables during the study in the liquorice group compared to the control group. In Figures 11, 12, and 14, the P-values refer to the within-group changes between the study visits. The other statistical results are presented in the text.

5.2.1 Baseline characteristics of the subjects

Studies II and III

The demographic and clinical characteristics of the study subjects are shown in Table 10. No differences were detected in these variables between the liquorice group and the control group in studies II-III.

In study III, the baseline 15-min haemodynamic recordings showed no statistically significant differences in radial systolic or diastolic BP (Figure 8), HR (Figure 9), or aortic FWA, PP, and AIx (Figure 10) between the groups. However, the upright decrease of CI was more pronounced in the liquorice group compared to the controls (ANOVA for repeated measurements time*group interaction $p=0.001$) at baseline (Figure 9). Also, a higher increase of SVRI during orthostatic challenge was observed in the liquorice than in the control group during the baseline measurements, while a significant time*group interaction (supine-upright-supine; $p=0.016$ at baseline) was detected during the 15-min recordings (Figure 9), but not during the initial 10-min of recordings (supine-upright; $p=0.075$ at baseline) (III).

Study IV

The control group included more men than the liquorice group, and the subjects were considerably older in the control than the liquorice group (Table 10). Also, waist circumference of both male and female subjects was higher in the controls, and several plasma variables differed between the groups (Table 10). During the baseline 15-min haemodynamic recordings performed without the research drugs, the level of aortic systolic and diastolic BP ($p<0.001$ for both, data not shown), HR ($p=0.005$) (Figure 11a and 11b), and SVRI ($p=0.037$) (Figure 12a and 12b) were higher in the control than in the liquorice group. Also, the mean (SD) PWV was 8.7 (1.1) m/s in the control group vs. 7.1 (0.7) m/s in the liquorice group at baseline ($p<0.001$ between the groups).

Table 10. Demographic and clinical characteristics of the subjects at baseline in studies II-IV. In statistical analyses, the liquorice group was compared to control group of studies II-III, or control group of study IV.

| | Control, n=29-30* (Studies II-III) | Control, n=20-21* (Study IV) | Liquorice, n=22 |
|----------------------------|---------------------------------------|---------------------------------|-----------------|
| Age (years) | 33.8 ± 8.1 | 47.3 ± 6.2° | 34.9 ± 9.2 |
| Male/Female | 13/17 | 18/3† | 8/14 |
| Waist circumference (cm) | | | |
| Male | 87 ± 4 | 103 ± 9° | 83 ± 7 |
| Female | 78 ± 10 | 92 ± 12† | 76 ± 7 |
| Haemoglobin (g/l) | 143 ± 15 | 151 ± 11° | 139 ± 9 |
| Fasting plasma | | | |
| Total cholesterol (mmol/l) | 4.4 ± 0.8 | 5.0 ± 0.8 | 4.6 ± 0.8 |
| LDL-cholesterol (mmol/l) | 2.2 ± 0.7 | 3.2 ± 0.7† | 2.4 ± 0.6 |
| HDL-cholesterol (mmol/l) | 1.72 ± 0.39 | 1.31 ± 0.28° | 1.83 ± 0.36 |
| Triglycerides (mmol/l) | 0.94 ± 0.49 | 1.38 ± 0.52° | 0.83 ± 0.32 |
| Glucose (mmol/l) | 5.0 ± 0.4 | 5.5 ± 0.5‡ | 5.2 ± 0.4 |
| Creatinine (µmol/l) | 76 ± 11 | 80 ± 13 | 77 ± 17 |
| Sodium (mmol/l) | 140 ± 2.0 | 141 ± 1.4† | 140 ± 1.3 |

Mean values ± standard deviations depicted.

*Blood samples for fasting plasma values were not obtained from one subject.

Mean value was significantly different from that of the liquorice group: °p<0.001, †p<0.01, ‡p<0.05, Independent samples t-test applied.

5.2.2 Effects of 2-week liquorice ingestion

5.2.2.1 Weight, renin and aldosterone levels, and urinary glucocorticoid excretion

The changes in weight and ECW volume after two weeks of liquorice ingestion are presented in Table 11 (**II**, **III**). In the liquorice group, weight was increased by 1.5 kg and ECW volume by 0.6 kg, while no significant changes were detected in the control group of studies II and III (between group p=0.001 and p<0.001, respectively). Further, plasma potassium (p=0.012) and aldosterone (p<0.001) concentration, and renin activity (p<0.001) were decreased within the liquorice group after two weeks (Table 11) (**IV**).

Before liquorice ingestion, the mean (SD) urinary THE excretion was 28.3 (18.9) nmol/l and allo-THF+THF 40.0 (24.1) nmol/l (**IV**). After the 2-week liquorice intervention urinary THE decreased by -16.9 nmol/l (95 % CI -25.9 to -7.9, p=0.001) and allo-THF+THF by -4.0 nmol/l (95 % CI -14.1 to 6.2, p=0.205). The individual values of ratio of THE to allo-THF+THF at week 0 and 2, and changes during the 2-week intervention are shown in Figure 13a. After two weeks of liquorice exposure, the mean reduction in ratio of THE to allo-THF+THF was -0.39 (-0.50

to -0.29, $p < 0.001$) as an indicator of the suppressed activity of the enzyme 11 β -HSD2.

Table 11. Changes in weight, extracellular water volume, plasma aldosterone, and plasma renin activity after two weeks of liquorice ingestion (studies II-IV)

| | Control n=29-30* | | Liquorice n=22 | | P value ^o |
|--------------------------------------|------------------|--------------|-------------------|--------------|----------------------|
| | Mean | SD (95 % CI) | Mean | SD (95 % CI) | |
| Weight (kg) | | | | | |
| Baseline | 71.2 | 8.9 | 69.6 | 9.2 | 0.431 |
| Change during the study | 0.1 | (-0.4; 0.6) | 1.5 | (0.8; 2.2) | 0.001 |
| Extracellular water (l) | | | | | |
| Baseline | 12.8 | 1.2 | 12.3 | 1.5 | 0.092 |
| Change during the study | -0.2 | (-0.4; 0.0) | 0.6 | (-0.0; 1.2) | <0.001 |
| Plasma potassium (mmol/l) | | | | | |
| Baseline | 3.9 | 0.2 | 3.9 | 0.3 | 0.658 |
| Change during the study | not analysed | | -0.2 [†] | (-0.4; -0.1) | - |
| Plasma aldosterone (pmol/l) | | | | | |
| Baseline | 840 | 1202 | 495 | 278 | 0.566 |
| Change during the study | not analysed | | -304 [‡] | (-434; -174) | - |
| Plasma renin activity (μ g/l/h) | | | | | |
| Baseline | 1.5 | 1.4 | 1.3 | 2.0 | 0.530 |
| Change during the study | not analysed | | -1.2 [‡] | (-2.1; -0.3) | - |

Mean values, standard deviations and 95 % confidence intervals depicted.

*Blood samples for fasting plasma values were not obtained from one subject.

^oMann-Whitney test.

[†] $p=0.012$ and [‡] $p<0.001$ for within-group comparisons, Wilcoxon signed ranks test applied.

5.2.2.2 Haemodynamics at rest

The mean changes in supine peripheral and central BP, aortic PP, and AIx after liquorice intake are depicted in Table 12 (II). Liquorice ingestion increased both radial and aortic systolic and diastolic BP by 7-8/4 mmHg, respectively ($p < 0.05$ for all within group comparisons). In addition, central PP was increased by 4 mmHg and AIx by 5 % ($p=0.018$ and $p=0.012$ within the liquorice group, respectively). In the control group, these haemodynamic variables were slightly, but not significantly, decreased during the follow-up. Between the liquorice and control group, the changes in BP, aortic PP, and AIx were statistically significant ($p < 0.05$ for all, Table 12). The change in weight did not correlate with changes in radial or aortic systolic/diastolic BP, aortic PP or AIx (data not shown). The changes in PWV during the study are presented in Table 13 (III). The within-group changes in PWV were small, but significantly different between the groups ($p=0.008$).

Table 12. Changes in blood pressure (BP), aortic pulse pressure, and augmentation index after two weeks of liquorice ingestion in the supine position (study II)

| | Control n=30 | | Liquorice n=20 | | P value* |
|------------------------------|--------------|--------------|----------------|--------------|----------|
| | Mean | SD (95 % CI) | Mean | SD (95 % CI) | |
| Radial systolic BP (mmHg) | | | | | |
| Baseline | 116 | 13 | 120 | 10 | 0.208 |
| Change during the study | -2.5 | (-5.5; 0.4) | 6.7 | (2.2; 11.3) | 0.001 |
| Radial diastolic BP (mmHg) | | | | | |
| Baseline | 64 | 10 | 68 | 7 | 0.106 |
| Change during the study | -0.3 | (-2.5; 1.9) | 4.4 | (0.5; 8.3) | 0.003 |
| Aortic systolic BP (mmHg) | | | | | |
| Baseline | 101 | 12 | 105 | 9 | 0.161 |
| Change during the study | -1.9 | (-4.5; 0.8) | 8.1 | (3.4; 12.8) | <0.001 |
| Aortic diastolic BP (mmHg) | | | | | |
| Baseline | 65 | 10 | 69 | 8 | 0.115 |
| Change during the study | -0.5 | (-2.7; 1.8) | 4.4 | (0.6; 8.3) | 0.018 |
| Aortic pulse pressure (mmHg) | | | | | |
| Baseline | 35 | 5 | 36 | 7 | 0.821 |
| Change during the study | -1.2 | (-2.6; 0.2) | 3.9 | (0.7; 7.0) | 0.005 |
| Augmentation index (%) | | | | | |
| Baseline | 13 | 10 | 14 | 10 | 0.899 |
| Change during the study | -0.9 | (-2.9; 1.1) | 5.1 | (1.3; 8.9) | 0.003 |

Mean values, standard deviations and 95 % confidence intervals depicted.

*Independent samples t-test

Table 13. Change in pulse wave velocity after two weeks of liquorice ingestion (study III)

| | Control n=30 | | Liquorice n=22 | | P value* |
|---------------------------|--------------|---------------|----------------|---------------|----------|
| | Mean | SD (95 % CI) | Mean | SD (95 % CI) | |
| Pulse wave velocity (m/s) | | | | | |
| Baseline | 7.1 | 1.1 | 7.1 | 0.7 | 0.208 |
| Change during the study | -0.18 | (-0.38; 0.03) | +0.27 | (-0.11; 0.65) | 0.008 |

*Mann-Whitney test.

5.2.2.3 Haemodynamics during orthostatic challenge

In study III, liquorice exposure increased radial systolic BP in both the supine and upright position, while the elevation of radial diastolic BP was observed during the first 5-min supine period (Figure 8). The HR response to upright posture was decreased after the 2-week liquorice intervention ($p=0.003$, Figure 9) (III). No significant differences were detected in the changes of CI during the study between the groups (Figure 9). However, SVRI was elevated after liquorice intake (Figure 9). Liquorice consumption also increased aortic FWA, PP, and AIx (Figure 10) (III). The elevation of AIx was pronounced during orthostatic challenge ($p=0.001$), and the FWA was increased in the upright position (Figure 10).

To examine the factors contributing to the observed liquorice-induced elevation of AIx, the correlations between changes in AIx, PWV, SVRI, HR, SV, ventricular ejection duration, and ECW volume were determined (III). The upright increase in AIx correlated with changes in HR ($r_s=-0.592$, $p=0.004$), ejection duration ($r_s=0.736$, $p<0.001$), and SVRI ($r_s=0.600$, $p=0.003$). No significant correlations were observed between changes in AIx and PWV, SV or ECW volume.

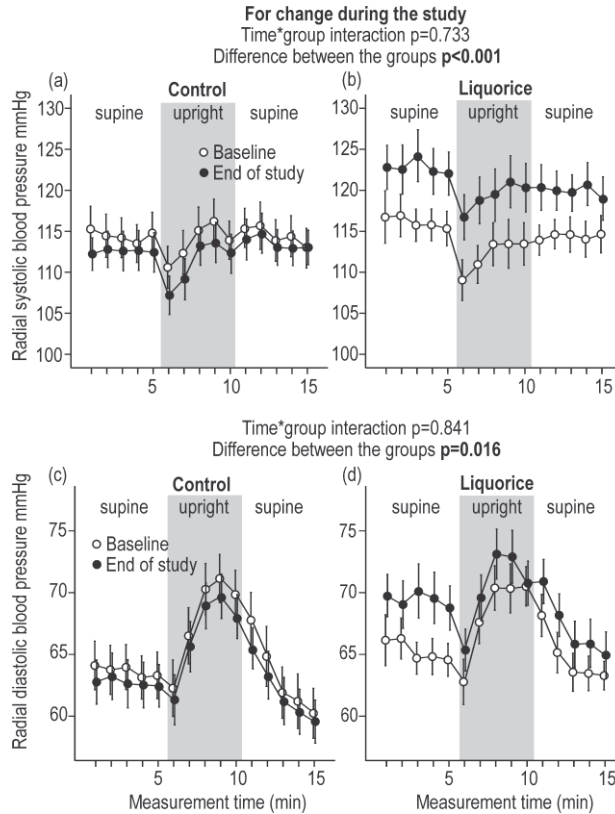


Figure 8. Radial systolic (a, b) and diastolic blood pressure (c, d) during the 15-min recordings at baseline and after the 2-week follow-up in the control group or intervention in the liquorice group (III). Mean and standard error of the mean represented. Statistical analyses compare the changes from baseline to the end of the study between the control and liquorice group during the measurement protocol.

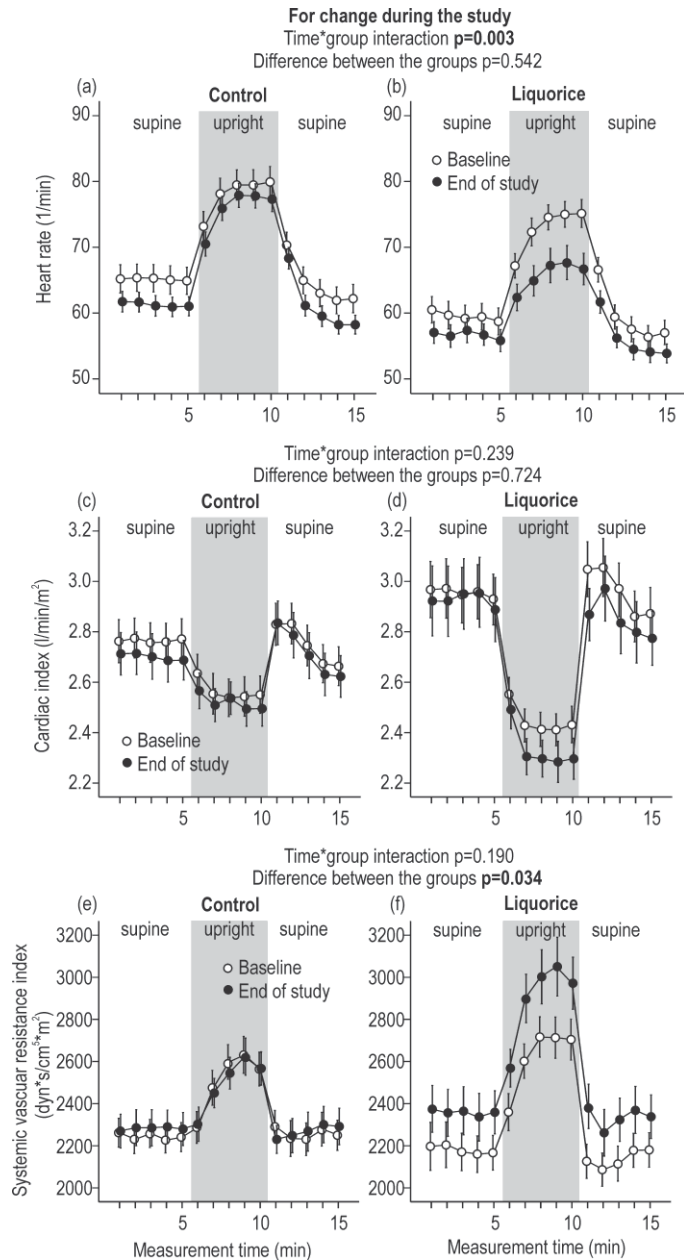


Figure 9. Heart rate (a, b), cardiac index (c, d), and systemic vascular resistance index (e, f) during the 15-min recordings at baseline and after the 2-week follow-up in the control group or intervention in the liquorice group (III). Mean and standard error of the mean represented. Statistical analyses compare the changes from baseline to the end of the study between the control and liquorice group during the measurement protocol.

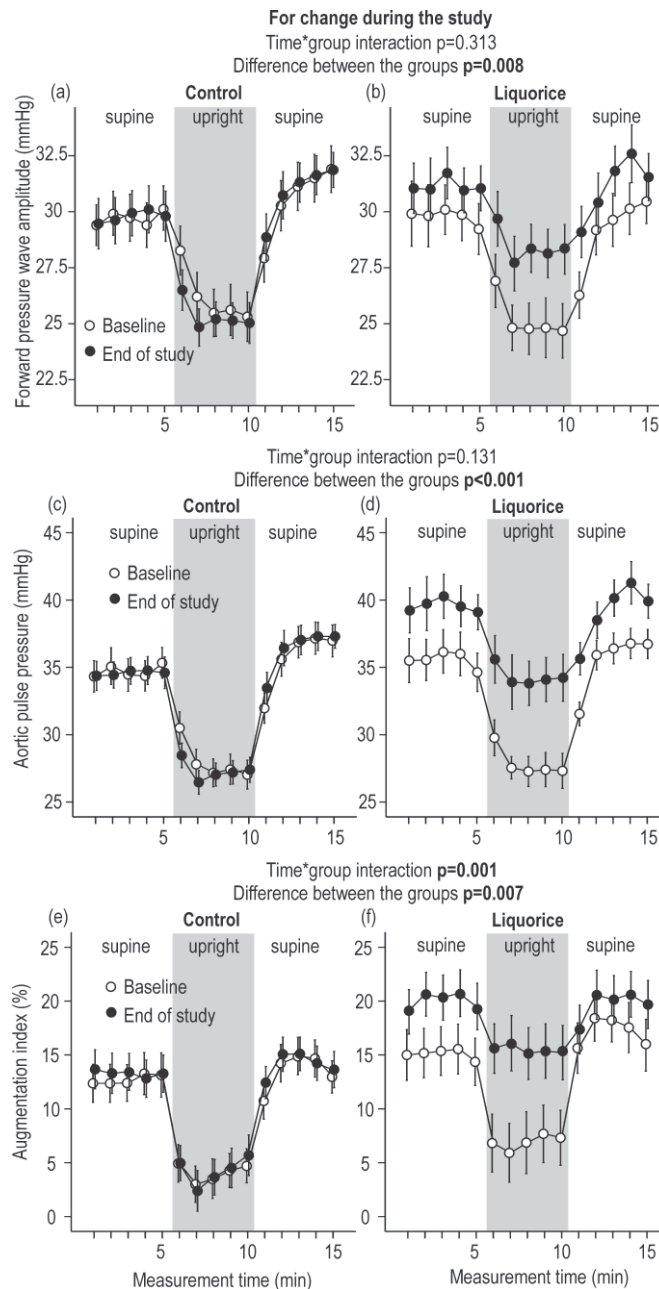


Figure 10. Forward pressure wave amplitude (a, b), aortic pulse pressure (c, d), and augmentation index (e, f) during the 15-min recordings at baseline and after the 2-week follow-up in the control group or intervention in the liquorice group (III). Mean and standard error of the mean represented. Statistical analyses compare the changes from baseline to the end of the study between the control and liquorice group during the measurement protocol.

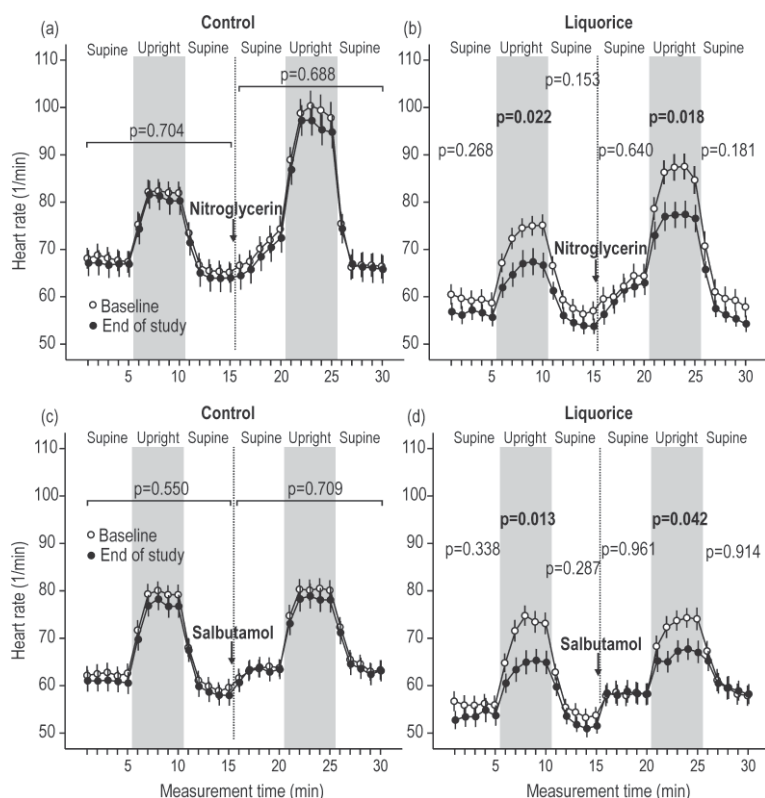


Figure 11. Heart rate at baseline and after the follow-up in the control group or 2-week intervention in the liquorice group during supine and upright position in study IV. Haemodynamic data was captured before (0-15 min) and after (16-30 min) administration of sublingual nitroglycerin 0.25 mg (a, b) and inhaled salbutamol 400 μ g (c, d). Mean and standard error of the mean represented. P-values refer to the differences between the study visits within the control and liquorice group.

5.2.2.4 Liquorice and haemodynamic responses to nitroglycerin and salbutamol

SVRI and AIx at baseline and after the control and liquorice diet in the absence and presence of nitroglycerin and salbutamol are shown in Figure 12 and 14, respectively (IV). During the control diet, no significant changes were observed in SVRI or AIx. Liquorice ingestion elevated SVRI before and after nitroglycerin administration during the upright and following supine position (Figure 12b) (IV). A moderate liquorice-induced increase in SVRI was also detected before salbutamol inhalation during the first supine and upright period (Figure 12d) (IV). However, following salbutamol administration there were no differences between the levels of SVRI

before and after liquorice intervention. The liquorice-induced increase in AIx observed before sublingual nitroglycerin was abolished after the drug administration (Figure 14b) (IV). In contrast, the liquorice-induced increase in upright AIx was observed in the absence and presence of salbutamol (Figure 14d).

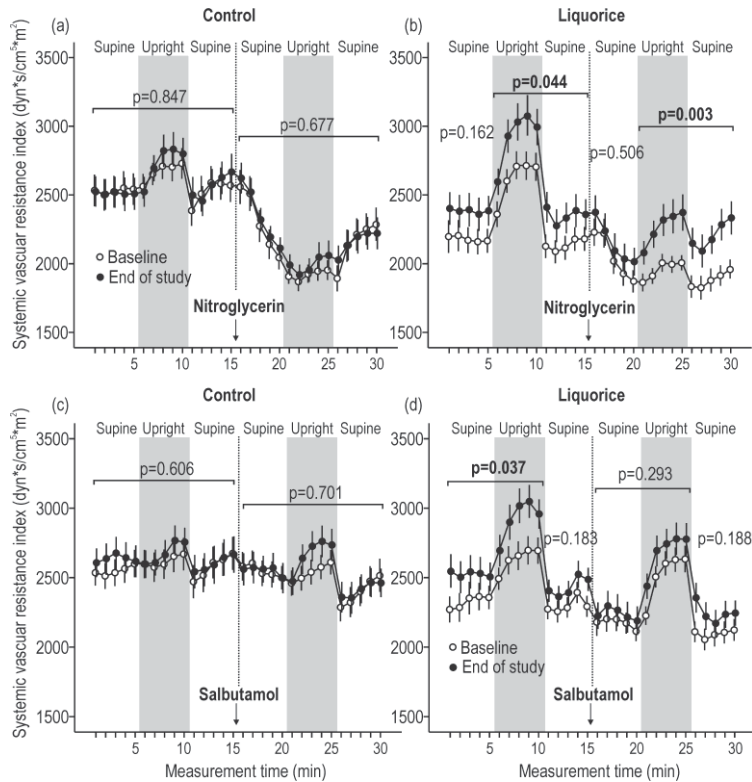


Figure 12. Systemic vascular resistance index at baseline and after the follow-up in the control group or 2-week intervention in the liquorice group before and after administration of sublingual nitroglycerin 0.25 mg (a, b) and inhaled salbutamol 400 µg (c, d) in study IV. Mean and standard error of the mean represented. P-values refer to the differences between the study visits within the control and liquorice group.

HR during the 15-min recording protocol before and after drug administration during the follow-up in the control and liquorice group are depicted in Figure 11 (IV). No significant changes were observed in HR during the control diet. In contrast, liquorice exposure induced consistent decrease in upright HR before and after nitroglycerin and salbutamol administration (Figure 11b and 11d). Furthermore, the change in cardiac chronotropic response correlated with the change in ratio of THE to allo-THF+THF ($r_s=0.475$, $p=0.026$, Figure 13b) (IV). No

correlations between the change in ratio of THE to allo-THF+THF and any other haemodynamic variables, weight, ECW volume, plasma aldosterone level and renin activity were detected.

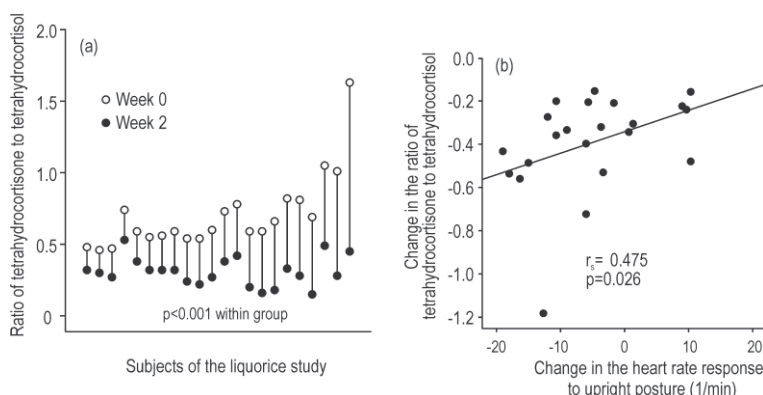


Figure 13. (a) The urinary ratio of tetrahydrocortisone to tetrahydrocortisol before and after liquorice intervention. Lines represent the magnitude of change in each individual and are arranged from the smallest to the highest. (b) The relation of the change in urinary ratio of tetrahydrocortisone to tetrahydrocortisol with the change in heart rate response to orthostatic challenge after liquorice intervention. Black circles represent individual values. r_s , Spearman's correlation.

5.2.2.5 Heart rate variability

The power spectral analyses of HRV at baseline and after two study weeks are presented in Figure 15 (III). No significant time*group interactions or within-groups changes were observed in the HF power or ratio of LF to HF in the supine or upright position ($p > 0.05$). The LF power was decreased after the 2-week liquorice intervention ($p = 0.034$), but no significant differences were detected between the groups. The upright ratio of LF to HF power was lower after 2 weeks of liquorice diet compared to control diet ($p = 0.008$).

5.3 The between group differences in functional haemodynamic regulation in studies I and III

The SVRI and CI responses to orthostatic challenge at baseline and end of the study I and study III are presented in Figure 16. In study I, increase in SVRI during

orthostatic challenge was higher in LTP5+PSe than LTP25+PSe group both at baseline and after 12 study weeks ($p<0.01$ and $p<0.05$ respectively). Also, the difference in supine to upright regulation of SVRI between the liquorice and control group persisted throughout the study III ($p<0.05$ and $p<0.01$, respectively). In addition, the greater upright decrease of CI in the liquorice than control group was observed both at baseline and week 2 ($p<0.01$ for both comparisons). In study I, the decrease in CI during orthostatic challenge was greater at week 0 than week 12 in the LTP5+PSe and placebo group ($p<0.05$ for both groups). In study III, no significant within group changes in SVRI or CI responses to upright posture were detected ($p>0.05$ for both groups).

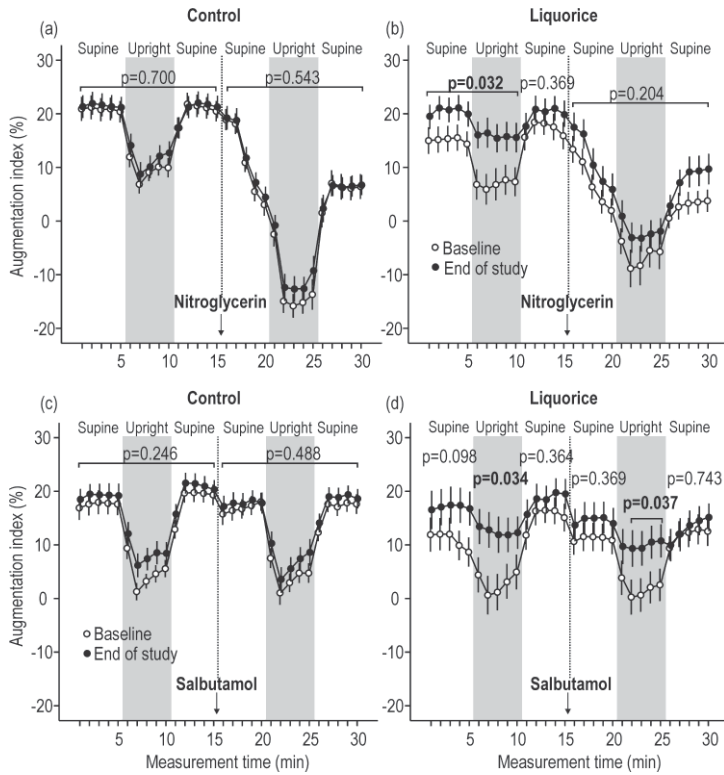


Figure 14. Augmentation index at baseline and after the follow-up in the control group or 2-week intervention in the liquorice group before and after administration of sublingual nitroglycerin 0.25 mg (a, b) and inhaled salbutamol 400 μ g (c, d) in study IV. Mean and standard error of the mean represented. P-values refer to the differences between the study visits within the control and liquorice group.

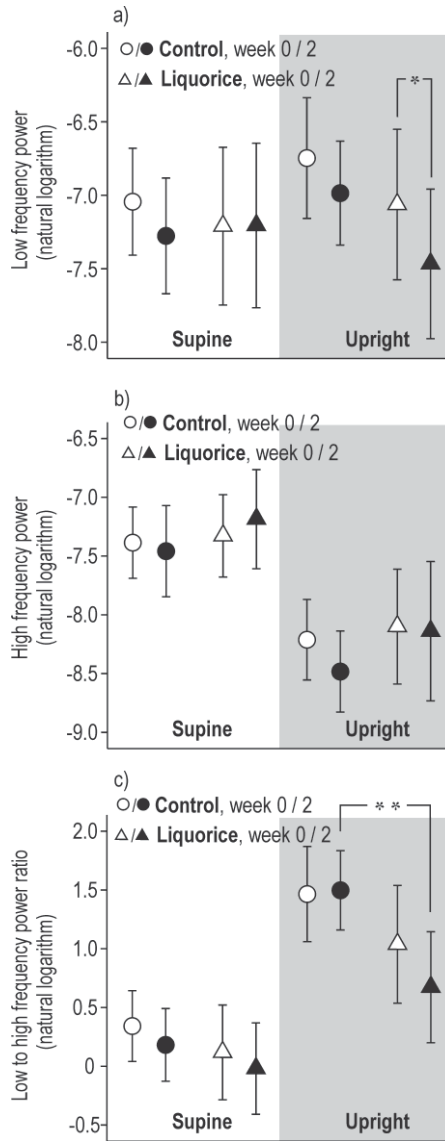


Figure 15. Low frequency power (a), high frequency power (b), and low to high frequency power ratio (c) in the supine and upright position (III). White circles and triangles represent the mean values (with 95 % confidence intervals by vertical bars) of the control and liquorice group at baseline, respectively, and black circles and triangles the corresponding values after 2 study weeks. * $p=0.034$ for the within-group difference between the visits, ** $p=0.008$ for the difference between the control and liquorice group in mean values after two study weeks; ANOVA for repeated measurements, and t-tests for post-hoc comparisons with Bonferroni corrections utilised.

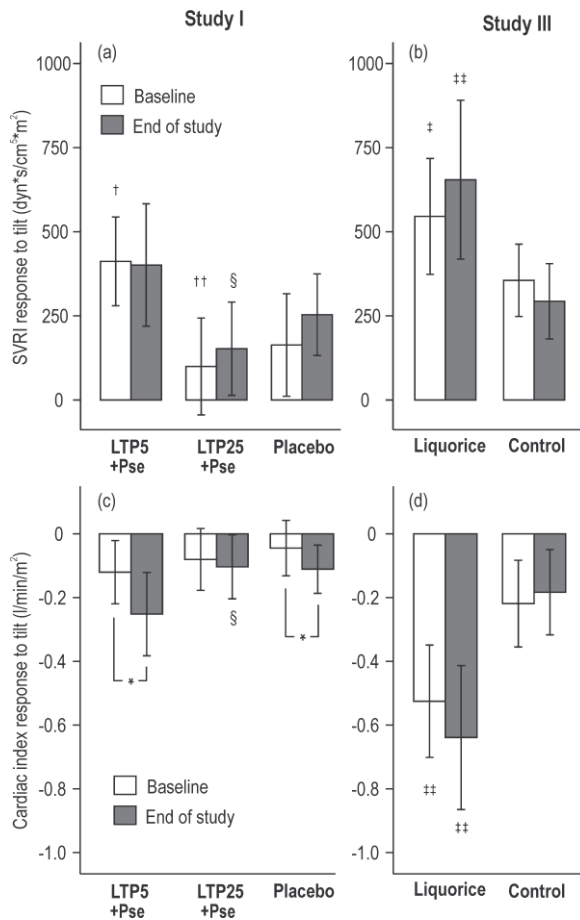


Figure 16. Haemodynamic responses to upright posture in systemic vascular resistance index (a, b) and cardiac index (c, d) during study I and III. Mean values and 95 % confidence intervals represented. Univariate ANOVA with age as covariate was utilised to compare mean values at baseline and at the end of study I between the groups with Bonferroni corrections in post hoc comparisons, while independent samples t-test was used in between-group comparisons in study III. Paired samples t-test was applied to test within-group differences between the study visits. [†]Mean value differed from that of the placebo group ($p < 0.05$). Mean value differed from that of the LTP5+PSe group: ^{††} $p < 0.01$ and [§] $p < 0.05$. Mean value differed from that of the control group: [‡] $p < 0.05$ and ^{‡‡} $p < 0.01$. Significant within-group difference: ^{*} $p < 0.05$. LTP5+PSe, 5 mg/d lactotripeptides and 2 g/d plant sterol esters; LTP25+PSe, 25 mg/d lactotripeptides and 2 g/d plant sterol esters.

6 DISCUSSION

6.1 Study subjects

The purpose of study I was to investigate the effect of combined intake of LTPs and PSe in fermented milk product on haemodynamics and plasma lipids in subjects with the MetS. One hundred and four individuals were included in the analyses of study I, and according to the mean baseline values of the study groups, the criteria of MetS were met with waist circumference ≥ 94 cm in men and ≥ 80 cm in women, office systolic and diastolic BP $\geq 140/85$ mmHg, respectively, and triglyceride concentration ≥ 1.7 mmol/l, and fasting plasma glucose ≥ 5.6 mmol/l (Table 6) (I) (Alberti et al. 2005). Although the subjects were randomised to one of the three study groups, the subjects in the LTP25+PSe group were older compared to the LTP5+Pse and placebo group (Table 6) (I). This is a limitation of study I, as the haemodynamic response to orthostatic challenge may differ between individuals under and over 50 years of age (Tahvanainen et al. 2009b). Aging increases arterial stiffness with subsequent increases in PWV, wave reflection, and central and peripheral systolic BP (McEniery et al. 2005; O'Rourke and Hashimoto 2007). In order to diminish the effect of different age distribution on the results, the statistical analyses were adjusted with age.

The studies II-IV consisted of a liquorice group of 20-22 healthy volunteers and two different control groups of 30 and 21 subjects in order to explore the haemodynamic alterations leading to elevation of BP after liquorice exposure. In studies II and III, the liquorice-induced alterations were compared with age-matched control subjects. The groups in studies II and III were homogenous with respect to age, body mass index (BMI) and sex distribution (Table 10), the factors that are known to influence haemodynamic characteristics of the subject (Franklin et al. 1997; Kangas et al. 2016). Further, the individual degree of liquorice-induced side effects is influenced by the rate of metabolism of glycyrrhizic acid to glycyrrhetic acid, and the absorption of the active metabolite depending on gastrointestinal transit time (Ploeger et al. 2001; Armanini et al. 2003). Also, the effects of liquorice intake may differ between men and women (Sigurjonsdottir et al. 2003; Sigurjonsdottir et al. 2006b; Sigurjonsdottir et al. 2006a), and the elderly may be more

sensitive to the harmful effects of liquorice intake (Omar et al. 2012; Nazari et al. 2017). However, the influences of liquorice consumption are mainly dependent on the ingested dose of glycyrrhizin (Sigurjonsdottir et al. 2001). The rather small number of the study subjects excluded comparisons between male and female subjects, but in future studies the liquorice-induced alterations on haemodynamics should also be compared between the sexes.

In studies I and III, the study groups showed differences in functional cardiovascular regulation of SVRI (Figure 16a and 16b) (**I**, **III**) and CI (Figure 16d) (**III**) during orthostatic challenge, which persisted throughout the study. Recently, the functional cardiovascular response to upright posture was divided into three distinct phenotypes, and the phenotype showing the lowest upright decrease in CI was also characterized with increased larger arterial stiffness (Tahvanainen et al. 2016). However, the effects of dietary interventions on upright cardiovascular regulation are largely unknown, thus the above-mentioned findings of studies I and III were not expected. In studies I-III, the subjects were without medications affecting BP or plasma lipids, which can be considered to strengthen the results of this study on haemodynamics.

As a limitation of study IV, the baseline demographic and clinical characteristics differed between the liquorice and control groups (Table 10). Also, three subjects in the control group were on statin treatment (Table 3) (**IV**), which has been suggested to lower both BP and arterial stiffness (Upala et al. 2017; Lamarche et al. 2018). Therefore, the effects of liquorice intake were not compared directly with the control group, but the changes during the liquorice intervention and control diet were analysed separately within the groups (**IV**). The purpose of the parallel investigation of haemodynamics in the control group was to examine whether the repeated measurements are characterized by systematic changes over time.

6.2 Study design and products

6.2.1 Study I

Study I was conducted as a randomised, double-blind, and placebo-controlled with 3 parallel groups. The aim of the 4-week run-in period was to introduce the study procedure to the subjects, after which the effects of intake of fermented milk products containing 5 mg and 25 mg LTPs combined with 2 g PSe daily were

examined during 12 weeks. According to previous investigations, the present doses of LTPs and PSe and the duration of the intervention period were adequate for detection of the possible antihypertensive (Xu et al. 2008; Cicero et al. 2011a; Qin et al. 2013; Turpeinen et al. 2013; Fekete et al. 2015) and lipid-lowering (Law 2000; Katan et al. 2003; Abumweis et al. 2008; Demonty et al. 2009; Musa-Veloso et al. 2011; Ras et al. 2014) influences. Further, the present design aimed to examine solely the effects of study products in the absence of lifestyle modifications. According to the unaltered body weight (Table 7), the background diet and exercise habits of the subjects remained constant throughout the study. Further, the reported compliance was excellent. The home BP measurements showed numerical reductions in all study groups including placebo (Table 8 and 9), and this phenomenon is often observed in trials investigating the effect of antihypertensive treatment (Wilhelm et al. 2016). Given the detected difference in the supine to upright regulation of SVRI between the LTP5+PSe and LTP25+PSe group (Figure 16a), a cross-over design would have been superior to a parallel design. However, an investigation exploring the long-term effects of two different interventions and placebo in over 100 subjects was more practical to conduct with the parallel design.

In study I, all study products including placebo were fermented milk products and no differences could be observed regarding to appearance and flavour. The LTPs were incorporated to the two test products as peptide powder, and plant sterols as esters of fatty acids from vegetable oil. The daily amount of protein and fat from the test products was slightly higher (+2 g for both) compared to the placebo product (Table 5). However, due to higher amount of carbohydrates (+7 g) in the placebo than the test products, the energy content of the study products was similar ~170 kcal. Further, the daily doses of calcium, potassium, and magnesium were higher in the test products when compared to the placebo: 380 vs. 250 mg, and 660 vs. 375 mg, and 41 vs. 28 mg, respectively (Table 5). The minerals are one putative explanation for the antihypertensive influence of milk intake (Van Mierlo et al. 2006; Kass et al. 2012; Binia et al. 2015), and therefore may contribute to the effects of both test and placebo milk product. However, in the present investigation no significant BP changes were detected between the study groups in spite of the higher mineral content of the test products.

6.2.2 Studies II-IV

The design of studies II-IV was open-label, which is a limitation of the present investigation. However, so far the manufacture of a liquorice product without glycyrrhizin but with authentic liquorice taste has not been possible, which excluded a double-blind cross-over design. Also, the present study aimed to examine the haemodynamic influences of commercial liquorice products, not pure glycyrrhizin, as this is the case in everyday life. The daily dose of glycyrrhizin (290-370 mg) was chosen so that it would not exceed 400 mg/d, a dose that induces harmful effects to most individuals (Stormer et al. 1993). Since the liquorice-induced rise in BP has been observed after glycyrrhizin ingestion of 75 mg/d within two weeks (Sigurjonsdottir et al. 2001), the length of the intervention and dosage of glycyrrhizin was considered sufficient for the detection of liquorice-induced alterations on haemodynamics.

Liquorice products also contain other components that might have an effect on cardiovascular function and health. Liquorice products are rich in carbohydrates originating from wheat flour, sugar and sugar syrup as ingredients. In studies II-IV, the average amount of carbohydrates from the liquorice products was 150 g/d. An increase in overall dietary glycaemic index has been associated with increased level of lipid peroxidation markers (Hu et al. 2006). However, several flavonoids possessing antioxidant activity against lipid peroxidation have been detected from the extract of liquorice root (Vaya et al. 1997; Li et al. 2011; Fu et al. 2013), and the balance between formation of free radicals and antioxidant defence possibly resulting from liquorice ingestion is unknown.

Furthermore, salt in the liquorice products could contribute to the elevation of BP. Regarding the present liquorice products, Halva liquorice™ contained no added salt (www.halva.fi), while salt was added to the Kouvola liquorice™ so that the final product contained salt 0.6 g/100 g (www.kouvolanlakritsi.fi). Thus, the possible highest amount of additional salt was 1.8 g/d in studies II-IV. A clinical trial with subjects aged ≥ 60 years and baseline systolic/diastolic BP of 139/78 mmHg found that daily salt increase of 2.9 g for 14 days in addition to low-salt diet (2.3 g salt per day) elevated systolic/diastolic BP by 6/0.3 mmHg (Johnson et al. 2001). A recent meta-analysis suggested that when compared with a salt dose of <5.2 g/d, no significant increase was detected in systolic/diastolic BP at a salt dose of 9.1-14.3 g/d in individuals with BP $<130/80$ mmHg, while a significant dose-response relation was detected in hypertensive individuals (Graudal et al. 2015). This meta-analysis included studies, in which the mean age ranged from 21 to 69 years (Graudal

et al. 2015). It seems unlikely, that in the present healthy and quite young population with mean baseline BP of 119/70 mmHg, the above-mentioned amount of salt would have significantly influenced the results.

6.3 Haemodynamic measurements

In the present series of studies, haemodynamics were recorded utilising ICG_{WB} recordings and pulse wave analysis from the radial artery at rest and during orthostatic challenge. In addition to these haemodynamic recordings in the laboratory, BP was measured at home by the study subjects during study I. Further, sublingual nitroglycerin and inhaled salbutamol were administered during the haemodynamic recording protocol in study IV to investigate possible alterations in the mechanisms of vasodilatation induced by exogenous NO or β_2 -adrenoceptor stimulation after liquorice exposure.

The ICG_{WB} is a non-invasive method to evaluate CO based on the measurements of SV and HR, and it calculates SVR based on CO and BP (Kööbi et al. 1997a; Kööbi et al. 1997b; Cotter et al. 2004). Previously, a good agreement has been demonstrated between the CO values obtained by the ICG_{WB} method and invasive thermodilution measurement both at rest and during orthostatic challenge (Kööbi et al. 1997a; Kööbi et al. 1997b), and the ICG_{WB}-derived SV correlates well with the 3-dimensional echocardiography recordings (Koskela et al. 2013). Furthermore, measurement of PWV by the ICG_{WB} has been validated against Doppler ultrasound (Kööbi et al. 2003) and SphygmoCor® applanation tonometry (Wilenius et al. 2016) with good correlation between these methods. In the present study, central BP and indices of central wave reflection were evaluated by radial applanation tonometry using an automatic sensor that was fixed with a wrist band on the radial artery (Tahvanainen et al. 2009a). This enabled the continuous detection of radial pulse wave producing beat-to-beat data, which is an advantage compared to the commonly used method recording only 10-20 pulse waves (Hayward et al. 2002).

A good reproducibility and repeatability has been demonstrated for the haemodynamic recording protocol used in the present study (Tahvanainen et al. 2009a). The utilisation of passive head-up tilt enabled the detection of differences in cardiovascular regulation between the study groups (**I**, **III**) and the pronounced effects of liquorice intake in the upright position (**III**, **IV**). In study IV, an adequate inhalation technique of salbutamol was guided by the study nurse in order to ensure the good delivery of salbutamol. After inhaled salbutamol, the plasma concentrations

have been demonstrated to increase from the first minute and reach a stable level between 5 to 20 min (Wilkinson et al. 2002). Thus, the present measurement period of 15 min can be considered sufficient for the detection of salbutamol-induced haemodynamic changes.

In study I, home BP measurements were performed according to ESC/ESH guidelines (Williams et al. 2018). These out-of-office BP measurements provided an additional method to investigate the possible antihypertensive effect of LTPs, strengthening the reliability of the results. Home BP monitoring gives more reproducible data compared to office brachial BP measurements, and values measured at home are usually lower (Williams et al. 2018). This was seen in study I, as the average baseline office systolic/diastolic BP for the whole study population was 163/100 mmHg, while the corresponding value was considerably lower, 142/88 mmHg, when measured at home at week 0 (I). Furthermore, during the continuous 5 min recordings of radial systolic and diastolic BP in the supine position, the baseline values seemed to be somewhat lower compared to the home BP measurements (Figure 6, Table 8).

6.4 Results and findings

6.4.1 Effect of fermented milk product containing lactotripeptides and plant sterol esters on blood pressure

Neither of the fermented milk products containing LTPs 5 mg/d or 25 mg/d with PSe 2 g/d lowered BP compared to controls in subjects with the MetS (Figure 6) (I). The present finding was observed in measurements performed both in the laboratory and at home. Although a recent meta-analysis indicated a significant reduction in systolic/diastolic BP (-2.95/-1.51 mmHg, respectively) after LTP consumption, the results of office and out-of-office measurements were not consistent (Fekete et al. 2015). When the analysis was restricted to 24-h ambulatory BP recordings, the antihypertensive effect of LTPs was markedly attenuated (-0.94/-0.46 mmHg for reduction in systolic and diastolic BP, respectively, $p > 0.05$ for both) (Fekete et al. 2015). However, in the meta-analysis of Qin et al. (2013) 24-h ambulatory BP measurements showed a small but statistically significant LTP-induced reduction in systolic (-1.3 mmHg) but not in diastolic (-0.57 mmHg) BP. Consistent with the present results, previous studies in Dutch (Engberink et al. 2008; Van Der Zander

et al. 2008), Scottish (Van Mierlo et al. 2009), and Danish (Usinger et al. 2010b) populations indicated no significant antihypertensive effect for LTPs. Although meta-analyses have shown a greater BP-lowering effect in Japanese (Chanson-Rolle et al. 2015) than European subjects (Cicero et al. 2013) after LTP-product consumption, the slight change in European populations was statistically significant. Explanations including differences in the background diet and body weight have been suggested for the inconsistency between Japanese and European studies (Fekete et al. 2015). Interestingly, BMI has been proposed to correlate negatively with the antihypertensive efficacy of LTPs (Fekete et al. 2015). In addition, in the meta-analysis by Cicero et al. (2013), age was associated with the magnitude of BP reduction so that the treatment effect on systolic BP was reduced by 0.09 mmHg with every additional year. The authors suggested that this might arise from isolated systolic hypertension with reduced response to first-line antihypertensive treatment characteristic to the elderly (Cicero et al. 2013). Thus, it can be speculated whether the mean age of 51 years and BMI of 31 kg/m² of the present study population attenuated the possible antihypertensive effect of LTPs.

Another factor that needs to be considered regarding the absence of BP-lowering effect of the present LTP-products is the simultaneous consumption of PSe. In SHR, combined treatment with LTPs and PSe attenuated the elevation of BP, but the treatment effect in comparison to controls was more pronounced after intake of LTPs alone than combined with PSe (Jäkälä et al. 2009b). Furthermore, phytosterols have been found to elevate systolic BP in salt-loaded stroke-prone SHR (Ogawa et al. 2003). In humans, plant sterols combined with minerals in low-fat and low-salt meat products had no effect on BP (Tapola et al. 2004). However, intake of spread containing LTPs (4.2 mg/d) and PSe (2 g/d) for 10 weeks reduced home systolic BP, while no significant changes were observed in office brachial BP, central BP, or 24-h ambulatory BP (Turpeinen et al. 2009; Turpeinen et al. 2012). In addition, fermented milk product containing LTPs (25 mg) and PSe (2 g) decreased BP after 8 hours of single oral dose (Turpeinen et al. 2011).

The anticipated outcome after consumption of products with possible *in vivo* ACE-inhibitory activity would be an increase in plasma renin activity and potassium concentration, and a decrease in plasma aldosterone concentration, as has been repeatedly observed during usage of ACE-inhibitory drugs (Macfadyen et al. 1993; Azizi et al. 1995; Alderman et al. 2012). However, the present study I did not detect significant changes in the aforementioned variables (Table 7) nor in the level of BP between the groups (I). The competitive ACE-inhibitory activity of LTPs has been documented *in vitro* (Nakamura et al. 1995a) and in animal experiments *in vivo*

(Nakamura et al. 1996; Sipola et al. 2002a). In contrast, this mode of action of LTPs has not been confirmed in humans (De Leeuw et al. 2009; Wuerzner et al. 2009; Boelsma and Kloek 2010; Usinger et al. 2010a), in accordance with the results of the present study. Although Ile-Pro-Pro has been shown to reach the circulation intact after LTP-product consumption (Foltz et al. 2007), the detected plasma concentration was lower than is required for effective ACE-inhibitory action (Nakamura et al. 1995a). Thus, the bioavailability of LTPs for ACE-inhibitory activity might be limited.

Other mechanisms contributing to BP-lowering effects of LTPs have been suggested including opioid-like activity (Nurminen et al. 2000; Sipola et al. 2002b) and inhibition of the sympathetic nervous tone (Usinger et al. 2010a). In the study of Usinger et al. (2010a) plasma noradrenaline was decreased in response to orthostatic challenge after consumption of fermented milk product containing LTPs for 8 weeks. However, no significant LTP-induced changes were detected in BP, HR, HRV, or SVR response to upright posture (Usinger et al. 2010a). Furthermore, SV and cardiac contractility has been found to decrease after 6 weeks of intake of LTP-product, while no changes were observed in CO or SVR (Cicero et al. 2011b). In study I, intake of LTPs had no significant effect on BP (Figure 6), CI or SVRI (Figure 7) in the supine or upright position. In the placebo and LTP5+PSe group, a numerical increase in CI, but decrease in SVRI was seen especially at rest (Figure 7) (I), which probably reflects the reciprocal relationship between CI and SVR (Guyton and Hall 2011).

6.4.2 Effect of fermented milk product containing lactotripeptides and plant sterol esters on plasma lipids

The present reduction of 0.1 mmol/l in LDL-cholesterol concentration after test product consumption (Table 10) (I) was modest when compared to a mean reduction of 0.34 mmol/l with 2.15 g/d of phytosterols in a previous meta-analysis (Demonty et al. 2009). The efficacy of PSe incorporated into low-fat milk products has been demonstrated (Clifton et al. 2004). Further, higher baseline level of plasma LDL-cholesterol (Demonty et al. 2009), and division of the daily dose of PSe over the day rather than ingestion of a single dose (Abumweis et al. 2009) have been proposed to enhance the treatment effect. In study I, the subjects had a moderately increased baseline level of LDL-cholesterol (mean 3.5 mmol/l) and the study products were ingested twice a day (in the morning and evening). These features may

have supported the cholesterol-lowering action of PSe. Further, a greater effect has been proposed when PSe-product is consumed simultaneously with a meal (Doornbos et al. 2006). In the meta-analysis of Abumweis et al. (2008), a single morning dose did not reduce LDL-cholesterol significantly, while PSe-product consumption once a day at lunch or evening meal resulted in a significant treatment effect.

The cholesterol-lowering effect of phytosterols has been previously shown in subjects with the MetS (Plat et al. 2009; Sialvera et al. 2012). However, a study including individuals with and without the MetS indicated no significant changes in plasma lipids in MetS patients, while significant reductions were observed in non-MetS subjects after consumption of low fat milk containing PSe (2 g/d) for 3 months (Hernandez-Mijares et al. 2011). Insulin resistance is known to associate with decreased intestinal cholesterol absorption (Simonen et al. 2000; Pihlajamaki et al. 2004), which may result in limited action and efficacy of PSe (Hernandez-Mijares et al. 2011), but also controversial results have been demonstrated in MetS (Plat et al. 2009; Sialvera et al. 2012).

6.4.3 Effects of 2-week liquorice ingestion

6.4.3.1 Weight, renin and aldosterone levels, and urinary glucocorticoid excretion

Salt and water retention, and decreased plasma concentrations of potassium and aldosterone, and reduced plasma renin activity are typical consequences of excessive liquorice ingestion (Epstein et al. 1977a; Farese et al. 1991; Armanini et al. 1996). In accordance with previous results, the series of studies II-IV demonstrated the syndrome of pseudohyperaldosteronism after two weeks of daily liquorice ingestion (Table 11). In addition, the urinary THE excretion and ratio of THE to allo-THF+THF was decreased (Figure 13a) (**IV**), which documents the inhibition of 11 β HSD2 and diminished metabolism of cortisol (Farese et al. 1991; Hammer and Stewart 2006). The enhanced activation of the renal MR by cortisol is a well-known mechanism leading to increased sodium and water reabsorption and subsequent elevation of BP during 11 β HSD2 deficiency (Ferrari 2010), but the GR is also expressed in the kidneys and might have a role in the regulation of sodium reabsorption and glucocorticoid-induced hypertension (Goodwin and Geller 2012; Hunter and Bailey 2015). The present liquorice ingestion resulted in 1.5 kg increase in body weight during the 2-week intervention most likely related to water retention

(Table 11) (**II**, **III**). Both enhanced action of endogenous cortisol and increased intake of carbohydrates following liquorice product ingestion could have influenced water balance. The parallel increase in ECW volume was about 0.6 kg (Table 11) (**II**, **III**), while the possible changes in the intracellular water volume were not determined in the present study. However, the increase in body weight did not correlate with changes in BP after liquorice exposure, in accordance with results reported by Sigurjonsdottir et al. (2003).

6.4.3.2 Haemodynamic variables

CO and SVR

The elevation of BP due to liquorice intake is well described in the literature (Conn et al. 1968; Farese et al. 1991; Sigurjonsdottir et al. 1995; Armanini et al. 1996; Sigurjonsdottir et al. 2001; Sigurjonsdottir et al. 2003), but the detailed haemodynamic changes after regular liquorice exposure are unknown. Liquorice intake increased the level of SVR, while no significant changes were observed in CO (Figure 9) (**III**). Both forms (1 and 2) of the enzyme 11 β HSD are expressed in the vascular wall, and by regulating the local glucocorticoid availability, these enzymes could influence vascular tone (Hadoke et al. 2006). However, the deficiency of 11 β HSD2, but not that of 11 β HSD1, affected vascular function in mice (Hadoke et al. 2001). The MR located both in the EC and VSMC has been demonstrated to regulate vascular tone and BP (Nguyen Dinh Cat et al. 2010; McCurley et al. 2012). During inhibition of 11 β HSD2, increased vasoconstriction may result from cortisol signalling via both the MR and GR (Hatakeyama et al. 2000; Hadoke et al. 2001). The effects on vascular function can be mediated via several mechanisms as inhibition of the 11 β HSD2 has enhanced aortic contractile responses to noradrenaline and ET-1, and suppressed the eNOS (Hadoke et al. 2001; Quaschnig et al. 2001; Ruschitzka et al. 2001). In addition, decreased activity of the 11 β -HSD2 enhanced cortisol-induced increase in Ang II binding in human VSMCs (Hatakeyama et al. 2000).

Nitroglycerin is a known exogenous NO donor inducing an endothelium-independent vasodilatation, while the vascular relaxation in response to β_2 -adrenoceptor agonist salbutamol is mediated via both endothelium-derived NO and via a direct effect on the VSMC (Ferro et al. 2004). Both of these drugs have been shown to decrease SVR, although the effect of nitroglycerin was clearly more pronounced (Tahvanainen et al. 2012). In the present study, the liquorice-induced

increase in SVRI was also observed after nitroglycerin administration, while under the influence of salbutamol no differences were detected between the SVRI level at weeks 0 and 2 (Figure 12) (IV). Thus, the study IV suggested that liquorice ingestion elevated SVR through impaired response to NO, while the vasodilatory response to β_2 -adrenoceptor stimulation remained unchanged during the intervention. In accordance with this, daily intake of 130 mg glycyrrhetic acid for 2 weeks attenuated endothelium-independent vasodilatation, while the effect on endothelium-dependent vascular relaxation was less clear in healthy humans (Sobieszczyk et al. 2010). In the study of Sobieszczyk et al. (2010) the vascular function was evaluated by forearm blood flow response to verapamil (endothelium-independent agent) and methacholine (endothelium-dependent agent). However, the previous results are partly conflicting, as both endothelium-independent and -dependent vasodilatation was impaired in 11 β -HSD2 knockout mice (Hadoke et al. 2001), while only the endothelium-dependent vascular relaxation was attenuated in rats that were fed glycyrrhetic acid (Quaschnig et al. 2001; Ruschitzka et al. 2001).

Aortic PP and AIx

The increase in aortic PP and AIx after liquorice exposure demonstrated that central wave reflection from the peripheral circulation was enhanced (Table 12, Figure 10) (II, III) (Laurent et al. 2006). Furthermore, the liquorice-induced elevation in AIx was detected especially in the upright position, that is, the upright AIx was decreased less during head-up tilt after liquorice intake (Figure 10f) (III). The increased AIx during head-up tilt was associated with parallel changes in HR, SVRI, and ejection duration (III), the variables which have been indicated to influence the magnitude of AIx (Kingwell and Gatzka 2002; Laurent et al. 2006; Wilenius et al. 2016). A parallel reduction of about 4 % has been observed in AIx with every 10 beats/min increment in HR (Wilkinson et al. 2002).

In study IV, the liquorice-induced effects on AIx were further investigated in the presence of nitroglycerin and salbutamol. Previously, the salbutamol-induced changes in AIx have been used for the evaluation of endothelium-mediated alterations in vascular tone (Wilkinson et al. 2002). However, the main reason for the reduced AIx after salbutamol inhalation may be the simultaneous increase in HR, as recently reported (Tikkakoski et al. 2018). After liquorice exposure, the upright AIx remained elevated in the presence of salbutamol (Figure 14d) although the level of SVRI was reduced (Figure 12d) (IV). This was probably due to the liquorice-induced impairment in cardiac chronotropic response to upright posture. However,

the influences of nitroglycerin on AIx and SVRI following liquorice ingestion were contrary to those of salbutamol (Figure 14b and 12b, respectively) (IV). Since the level of AIx is significantly associated with SVR (Wilenius et al. 2016), the strong vasodilatory effect of nitroglycerin may have blunted the liquorice-induced elevation of AIx (Figure 14b) (IV). Furthermore, the haemodynamic effects of inhaled salbutamol and sublingual nitroglycerin have been found to associate with body position as salbutamol-induced haemodynamic influences were more pronounced in the supine position, while those of nitroglycerin were accentuated during orthostatic challenge (Tahvanainen et al. 2012).

Indices of arterial stiffness

Assessment of PWV is a generally accepted, reproducible method to evaluate arterial stiffness (Laurent et al. 2006). PWV was slightly increased after liquorice ingestion, while a small reduction was observed during follow-up in the controls, and these changes were significantly different between the groups (Table 13) (III). As a slight effect was detected in PWV, further evaluations were made by measuring aortic FWA, and an increase was observed in the upright position after liquorice ingestion (Figure 10b) (III). This forward component of pressure wave has been proposed to increase as a result of mismatch between peak aortic flow and aortic root properties, and associate with increased aortic stiffness (Mitchell et al. 2004; Kaess et al. 2012). However, the interpretation of results must be done carefully as the amplitude of forward pressure wave may also be influenced by backward waves reflected from the periphery (Phan et al. 2016). Nevertheless, the moderate changes in PWV and FWA in response to liquorice ingestion suggest that even a quite short period of regular liquorice exposure may increase large arterial stiffness. Although the MR signalling in the VSMCs may lead to remodelling of the vascular wall (Tarjus et al. 2015), structural changes are expected to develop during longer period of time. Thus, the probably reason for the observed increase in large arterial stiffness within 2 weeks was the liquorice-induced elevation of BP, as the prevailing BP distending the vessels has a strong influence on the indices of arterial stiffness (Laurent et al. 2006).

HR and HRV

Liquorice ingestion attenuated the cardiac chronotropic response to upright posture (Figure 9) (III). Thus, interestingly, the liquorice-induced decrease in HR was observed in the upright but not in the supine position. In parallel with reduced upright HR, power spectral analyses of HRV demonstrated reduction in LF power

(Figure 15a) (III), the component that predominantly correlates with sympathetic activity (Xhyheri et al. 2012). Further, the ratio of LF to HF power reflecting sympathovagal balance (Xhyheri et al. 2012) was lower in the upright position after 2 weeks of liquorice intake compared to controls (Figure 15c) (III). A factor that might have influenced the present finding of impaired cardiac chronotropic response to upright posture is increased ECW volume. In healthy subjects, ingestion of 500 ml of water 15 min before head-up tilt to 60° attenuated the increase in HR during orthostatic challenge (Schroeder et al. 2002). Although we found no changes in HF power (Figure 15b), Schroeder et al. (2002) observed that the upright decrease of this variable was blunted following water ingestion, probably as a consequence of increased cardiac parasympathetic tone. Further, in the study of Schroeder et al. (2002), drinking of water induced a slight increase in mean BP and peripheral resistance in the supine but not in the upright position. Also, water ingestion was found to improve orthostatic tolerance due to improvement in systemic and cerebral haemodynamics, but increased plasma volume was not the only explanation for the effect (Schroeder et al. 2002).

Autonomic nervous system is a significant contributor to the regulation of BP (Navar 2014), but the results of the spectral analysis of HRV indicated that the liquorice-induced rise in BP was not related to changes in sympathovagal balance (Figure 15) (III). However, the enzyme 11 β -HSD2 and the MR are expressed in the brain and therefore might participate in the central regulation of BP and salt appetite (Chapman et al. 2013). In animal models, intracerebroventricular infusion of liquorice derivatives elevated BP (Gomez-Sanchez and Gomez-Sanchez 1992; Zhang et al. 2006), and HR and renal sympathetic nerve activity, which was reversed by a MR antagonist (Zhang et al. 2006). In addition, absence of 11 β -HSD2 in the brain has increased salt appetite, salt-sensitivity, and pressor responses to α_1 -receptor stimulation in mice (Evans et al. 2016).

Study IV further confirmed the finding of liquorice-induced impairment in cardiac chronotropic response during orthostatic challenge in the presence of nitroglycerin and salbutamol (Figure 11), as both of these drugs have increased the level of HR (Tahvanainen et al. 2012). The finding that decreased urinary ratio of cortisone to cortisol metabolites correlated with the reduced cardiac chronotropic response (Figure 13b) (IV) may suggest a direct action of cortisol on the heart. The literature indicates that glucocorticoids may induce direct effects on the heart via both the MR and GR (Richardson et al. 2016). In addition, some previous reports have demonstrated a direct action of glycyrrhizic acid, or glycyrrhetic acid or its derivative on the heart using *in vitro* or *in vivo* animal experiments, or silico approach.

The carbenoxolone has caused gap junction uncoupling and slowing of atrial and ventricular conduction in the heart (De Groot et al. 2003). In addition, in isolated perfused rat hearts, glycyrrhetic acid induced negative inotropic effect (Parisella et al. 2012), and carbenoxolone decreased HR (Howarth and Qureshi 2006). Furthermore, intraperitoneal administration of glycyrrhizic acid induced an acute reduction in mice HR, which was proposed to arise from a direct blockade of β -adrenergic receptors by glycyrrhizic acid (Singh et al. 2016). Thus, also a direct action of glycyrrhetic acid or its derivative on the heart might have contributed to the decrease in upright HR after liquorice exposure (IV).

6.5 Clinical implications

A healthy eating pattern has been shown to be effective in both lowering elevated BP and improving related metabolic risk factors (Appel et al. 1997; Sacks et al. 2001; Uusitupa et al. 2013; Brader et al. 2014; Domenech et al. 2014). As possible functional foods, milk casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro with possible ACE-inhibiting properties have been proposed as an alternative for the dietary treatment of prehypertension or mild hypertension (Cicero et al. 2013; Turpeinen et al. 2013). In the present study I, no signs of ACE-inhibition or changes in BP were detected in the subjects after consumption of LTP+PSe products. Thus, the results of the present study (I) did not support the possible BP-lowering action of LTPs and suggested no significant role for the LTPs in the dietary treatment of elevated BP. However, a slight LDL-cholesterol lowering action (-0.1 mmol/l) was observed following the intake of LTP+PSe products (I). According to previous meta-analyses, the LDL-cholesterol lowering efficacy of phytosterol intake has been -0.34 mmol/l or -8-10 % with doses of about 2 g/d (Demonty et al. 2009; Ras et al. 2014). Therefore, food products enriched with plant sterols or stanols have been considered possible components for the dietary treatment of hypercholesterolemia (Finnish Current Care Guidelines; Gylling et al. 2014). However, in study I including MetS subjects, the LDL-cholesterol lowering effect remained quite modest.

Excessive liquorice ingestion is a known reason for dietary-based hypertension (Farese et al. 1991). The present study II demonstrated that already two weeks of daily liquorice ingestion in healthy subjects resulted in significant elevation of supine systolic/diastolic BP about 7-8/4 mmHg, respectively. This level of elevation of BP is considerable, as during BP-lowering drug therapy the average reduction is about 9/6 mmHg in systolic/diastolic BP by one antihypertensive agent (Law et al. 2003).

The possible sources of glycyrrhizin are various and should be considered as a possible reason for elevated BP in hypertensive patients to avoid unnecessary medications. Furthermore, study IV showed that after liquorice intake the vasodilatory effect of sublingual nitroglycerin was attenuated. This should be taken into account especially in patients with cardiovascular disease requiring nitroglycerin treatment. Also, hypertensive patients are often more sensitive to the elevation of BP following liquorice intake (Sigurjonsdottir et al. 2003), and it is recommended that they should avoid consumption of glycyrrhizin-containing products (Finnish Heart Association).

An important finding of the present study was that the study groups presented with functional differences in the supine to upright regulation of SVRI (**I, III**) and CI (**III**), in accordance with a recent report (Tahvanainen et al. 2016). Therefore, when investigating the effect of dietary intervention on haemodynamics the study should be conducted with a cross-over design to reduce the possible confounding arising from the differences in the cardiovascular regulation. Furthermore, the effects of liquorice consumption were pronounced during orthostatic challenge as the enhanced central wave reflection and impaired cardiac chronotropic response were particularly detected in the upright position (**III, IV**). Thus, dietary interventions may have an effect on upright cardiovascular regulation, and utilisation of a cardiovascular reactivity test enables the detection of more detailed information about the dietary influences on haemodynamics.

7 SUMMARY AND CONCLUSIONS

The present study was carried out to investigate the effects of simultaneous intake of milk casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro previously suggested to inhibit the RAAS, and PSe known to decrease the absorption of cholesterol, on haemodynamics and plasma lipids in subjects with the MetS. Two doses of LTPs (5 and 25 mg/d) were studied in combination with PSe 2 g/d in a fermented milk product. In addition, the detailed haemodynamic changes underlying the liquorice-induced elevation of BP, which is characterised by the suppression of the RAAS due to inhibition of the enzyme 11 β HSD2 and enhanced action of endogenous cortisol, were investigated in healthy, normotensive volunteers. For this purpose, 120-300 g of liquorice was consumed daily (that is 290-370 mg/d glycyrrhizin) for two weeks.

The main findings and conclusions of the present study are:

1. In subjects with the MetS, long-term intake of fermented milk products containing LTPs and PSe showed no antihypertensive effect measured at home or in the laboratory, and no signs of ACE-inhibiting activity *in vivo* was observed. However, a mild LDL-cholesterol lowering effect was detected in response to test product consumption (**I**).
2. In healthy subjects, two weeks of regular liquorice intake elevated central and peripheral BP via multiple mechanisms including increased extracellular fluid volume, amplified pressure wave reflection from the periphery especially in the upright position, elevated peripheral arterial resistance, and increased large arterial stiffness (**II**, **III**).
3. The liquorice-induced increase in systemic vascular resistance was attributed to impaired response to exogenous NO but not to β_2 -adrenoceptor stimulation (**IV**).
4. Liquorice exposure decreased cardiac chronotropic response to upright posture (**III**), and this impairment sustained in spite of the administration of

sublingual nitroglycerin and inhaled salbutamol (**IV**). The attenuated cardiac chronotropic response to upright posture significantly correlated with the decreased urinary cortisone to cortisol metabolite ratio, a marker of the inhibition of the enzyme 11 β HSD2 (**IV**).

5. Measurement of supine haemodynamics only underestimates the influences of liquorice intake (**III**).
6. Subjects differ in their phenotypic response to upright posture, that is in the associated increase in SVRI and decrease in CI, – and these changes appear to remain quite constant at least for a period of 12 weeks (**I, III**).

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10 ORIGINAL PUBLICATIONS

PUBLICATION

I

Effect of fermented milk product containing lactotripeptides and plant sterol esters on haemodynamics in subjects with the metabolic syndrome – a randomised, double-blind, placebo-controlled study

Hautaniemi EJ, Tikkakoski AJ, Tahvanainen A, Nordhausen K, Kähönen M, Mattsson T, Luhtala S, Turpeinen AM, Niemelä O, Vapaatalo H, Korpela R, Pörsti IH.

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**Effect of fermented milk product containing lactotripeptides and
plant sterol esters on haemodynamics in subjects with the metabolic syndrome
– a randomized, double-blind, placebo-controlled study**

Short title: Lactotripeptides plus plant sterols in MetS

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Abstract

We investigated the effects of fermented milk product containing Ile-Pro-Pro, Val-Pro-Pro and plant sterol esters, on plasma lipids, blood pressure, and its determinants systemic vascular resistance and cardiac output. In a randomized, double-blinded, placebo-controlled study, 104 subjects with metabolic syndrome were allocated to three groups to receive fermented milk product containing i) 5 mg/d lactotriptides and 2 g/d plant sterols, ii) 25 mg/d lactotriptides and 2 g/d plant sterols, or iii) placebo for 12 weeks. Plasma lipids and home blood pressure were monitored, and haemodynamics examined in laboratory using radial pulse wave analysis and whole-body impedance cardiography, in supine position and during orthostatic challenge. There were no differences between the effects of the two treatments and placebo on blood pressure at home, or on blood pressure, systemic vascular resistance index, and cardiac index in the laboratory, neither in supine nor upright position. The changes in plasma low-density lipoprotein cholesterol concentration were -0.1 mmol/l (95% CI -0.3, 0.1 and -0.3, 0.0) in the 5 and 25 mg/d lactotriptide groups, respectively, versus +0.1 mmol/l (95 % CI -0.1, 0.3) during placebo ($p=0.024$). Both at baseline and week 12 the increase in systemic vascular resistance during head-up tilt was lower in the 25 than 5 mg/d lactotriptide group ($p<0.01$), showing persistent differences in cardiovascular regulation between these groups. In subjects with metabolic syndrome, intake of lactotriptides and plant sterol esters in fermented milk product showed a lipid-lowering effect of borderline significance, while no antihypertensive effect was observed at home or in the laboratory.

Introduction

Hypertension is a major risk factor for cardiovascular disease, and it often clusters with other risk factors including central obesity, dyslipidemia, and insulin resistance. Metabolic syndrome (MetS) is a combination of these cardiometabolic risk factors, and it is associated with increased risk of type 2 diabetes and atherosclerotic cardiovascular disease⁽¹⁾. The prevalence of MetS is about 20 % among adult population⁽¹⁾.

Lifestyle changes, like reduction of salt and saturated fatty acid intake, increase in low-fat dairy product, fruit and vegetable intake, weight reduction, and increase in physical activity, play a key role in the prevention and treatment of hypertension and related disorders⁽²⁾. The possible antihypertensive effect of dairy product ingestion has been linked to the intake of calcium, potassium and magnesium, minerals that exist in rich amounts in milk⁽³⁾, and to bioactive peptides released from milk protein through gastrointestinal digestion, microbial fermentation or enzymatic hydrolysis⁽⁴⁾.

Three recent meta-analyses reported that ingestion of milk casein-derived lactotripeptides (LTPs) isoleucine-proline-proline (Ile-Pro-Pro) and valine-proline-proline (Val-Pro-Pro) decreased systolic blood pressure (BP) by about 4-5 mmHg and diastolic BP by about 2 mmHg⁽⁵⁻⁷⁾. Another meta-analysis by Pripp⁽⁸⁾ included clinical trials on both LTPs and other peptides derived from food, and found a lowering effect on systolic/diastolic BP of about 5/2 mmHg. One suggested antihypertensive mechanism of Ile-Pro-Pro and Val-Pro-Pro was competitive inhibition of the angiotensin-converting enzyme⁽⁹⁾. However, contradictory data also exist, as several clinical trials reported that LTPs were without significant influences on BP⁽¹⁰⁻¹²⁾.

The cholesterol-lowering effect of phytosterols (plant sterols and stanols) is well-established, as these compounds inhibit the absorption of dietary and endogenous cholesterol and up-regulate intestinal cholesterol efflux transporters⁽¹³⁾. Daily intake of 2 grams of plant sterols or stanols has been reported to reduce total and low-density lipoprotein (LDL) cholesterol levels by approximately 10 %⁽¹⁴⁾. The efficacy of plant sterols/stanols is affected by subjects' baseline LDL levels, food carrier, and frequency and timing of sterol intake⁽¹⁴⁾.

Two long-term intervention studies have examined the effects of simultaneous intake of LTPs and plant sterol esters (PSe) on cardiovascular risk factors in hypertensive and hypercholesterolaemic subjects^(15,16). In these studies, the intake of 4.2 mg LTPs with 2 g PSe in a low-fat spread was reported to decrease systolic BP by 4-6 mmHg, and LDL cholesterol concentrations by 0.2-0.3 mmol/l. However, the BP-lowering effect was not consistent in all measurements performed at home, in the office, or during ambulatory 24-hour recordings^(15,16).

Since the evidence about the BP lowering properties of LTPs remains contradictory, we tested the hypothesis whether simultaneous intake of LTP and PSe in fermented milk product lowers BP in subjects with MetS. Two doses of Ile-Pro-Pro and Val-Pro-Pro (5 and 25 mg/d) were studied, and since concurrent reduction in plasma lipids can be anticipated to provide additional cardiovascular benefits both products also contained 2 g/d of PSe. For the first time the long-term effects on haemodynamics were examined in a clinical physiology laboratory in a double-blind randomized parallel group set-up.

Methods

Ethical statement

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of Tampere University Hospital (Study code R08012). Written informed consent was obtained from all subjects. The study is a part of an ongoing investigation, in which hemodynamics are noninvasively recorded (DYNAMIC-study; Clinical Trials registration number NCT01742702), and the recording protocol is also registered in the EU Clinical Trials Register (EudraCT-number 2006-002065-39).

Study subjects

Altogether 116 subjects aged 35-62 years with MetS were recruited through announcements in a local newspaper. Inclusion criteria were waistline ≥ 94 cm for men and ≥ 80 cm for women, systolic BP ≥ 140 mmHg or diastolic BP ≥ 85 mm, and at least one of the following: plasma triglycerides ≥ 1.7 mmol/l, high density lipoprotein (HDL) cholesterol concentration < 1.03 mmol/l in men and < 1.29 mmol/l in women, and fasting glucose ≥ 5.6 mmol/l. Exclusion criteria were use of antihypertensive or lipid-lowering medication, secondary hypertension, unstable coronary artery disease, diabetes, malignancy, milk allergy, smoking, alcohol abuse, pregnancy, and lactation.

One subject discontinued the study during the run-in, and three subjects on placebo and two on 25 mg LTP + 2 g PSe discontinued the study during the intervention due to personal reasons. Four subjects were excluded because they had failed to report the use of statin (n=1) or metformin (n=3) at entry, and two subjects due to technical problems during haemodynamic recordings. Altogether 104 subjects (63 male, 41 female) were included in the final analysis.

Design

This study was randomized, double-blind and placebo-controlled with parallel design containing two intervention groups on different peptide concentrations and a control group. Separate randomization lists were used for men and women. During a 4-week run-in period, commercial fermented milk product without the test substances was given twice daily to familiarize the subjects with the trial procedure. After run-in, the subjects received fermented milk product containing i) 5 mg/d LTPs + 2 g/d PSe (LTP5+PSe), ii) 25 mg/d LTPs + 2 g/d PSe (LTP25+PSe), or iii) placebo without added LTPs and PSe. The product dose was 125 ml twice daily during the 12-week intervention. The subjects were instructed to maintain their medication, lifestyle, and dietary habits constant during the study, and register the use of the test products to study diaries.

Medications, use of vitamins and other nutrient supplements, and lifestyle habits were reviewed by structured questionnaires. Routine laboratory tests were taken at screening. At weeks 0, 8 and 12, blood samples after approximately 12 h of fasting were drawn, and the subjects were weighed. Laboratory measurements included full blood count, C-reactive protein, glucose, creatinine, uric acid, calcium, phosphate (0 and 12 week), and plasma lipids, sodium, and potassium (0, 8 and 12 week). BP was measured at home twice a week during the intervention (see below), and haemodynamics were recorded at weeks 0, 8 and 12.

Study products

All study products were produced by Valio Ltd., Helsinki, Finland. The run-in product was a non-fat fermented milk product, different from the test and placebo products, without bioactive LTPs and PSe. During the intervention, the fermented milk products contained Ile-Pro-Pro and Val-Pro-Pro (5 mg/d or 25 mg/d) and plant sterol esters (2 g/d). LTPs were added to the test products as peptide powder. The peptides were separated from *Lactobacillus helveticus* Lc 1936 fermented milk, concentrated using nanofiltration, and dried to produce powder. PSe were prepared by esterification of free plant sterols with fatty acids obtained from vegetable oil (Cognis Corporation, Illertissen, Germany), and contained β -sitosterol max 80 %, campesterol max 40 %, stigmasterol max 30 %, β -sitostanol max 15 % and campestanol max 5 %. Other sterols and stanols were present at <5%. Placebo was a fermented milk product without LTPs and PSe. The daily 250 ml dose of both test products contained 3 g fat, 10 g protein, and 25 g carbohydrates, and that of placebo contained 1 g fat, 8 g protein, and 32 g carbohydrates. The energy content of the products was similar. The daily contents of calcium, potassium and magnesium in the test products were 380 mg, 660 mg and 41 mg, respectively, and in placebo 250 mg, 375 mg and 28 mg, respectively.

Laboratory analyses

Blood count was determined using ADVIA 120 or 2120 (Bayer Health Care, Tarrytown, NY, USA), and other laboratory values using Cobas Integra 700/800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland). Total cholesterol, HDL and triglycerides were measured enzymatically, and LDL was calculated using the Friedewald equation⁽¹⁷⁾. Plasma insulin was determined using electrochemiluminescence assay on Cobas e411 analyzer (Roche Diagnostics). Plasma renin activity was measured using GammaCoat Plasma Renin Activity assay (Diasorin). Aldosterone concentrations were analyzed by active aldosterone RIA (Diagnostics Systems Laboratories, Beckman Coulter). Estimated creatinine-based glomerular filtration rate (eGFR) was calculated using the RULE formula⁽¹⁸⁾, as the plasma creatinine values were within normal range.

BP measurements in the office and at home, and body weight

Brachial office BP was measured in duplicate using an automated sphygmomanometer (Omron M4, Omron Matsusaka Ltd., Matsusaka, Japan) after 5 min rest in the sitting position at screening and at the end of the intervention⁽²⁾. Height, waist and hip circumference were measured to the closest 0.5 cm at screening. Body weight with subject in light clothing was monitored using digital scale at every visit. After hands-on instructions, home BP (Omron M4) was measured twice a week (one weekday and one weekend day) in the morning approximately 1 h after waking up.

Pulse wave analysis (PWA) and whole-body impedance cardiography

Haemodynamic recordings using continuous radial PWA (SpygmoCor PWMx, Atcor medical, West Ryde, Australia) and whole-body impedance cardiography (CircMon^R, JR Medical Ltd., Tallinn, Estonia) were performed in a quiet, temperature-controlled laboratory as reported previously in detail^(19,20). The PWA probes in the left arm were at the level of the heart throughout the measurements. For five min the subjects were resting supine on the tilt table, followed by five min of head-up tilt to 60 degrees, and then the tilt table was returned to the horizontal position for five min. The electrode configuration⁽²¹⁻²³⁾, and the good repeatability and reproducibility of the measurements has been reported⁽¹⁹⁾. Caffeine containing products, smoking and heavy meal for at least 4 h, and alcohol for at least 24 h prior to the recording were to be avoided.

Statistical analyses

With sample size 35 per group, the present study had a 82 % power for detecting a 7 mmHg difference in systolic BP by using standard deviation 10, α -level 0.05 and power analysis method for two-sample t-test⁽²⁴⁾. Statistical analyses were performed using SPSS for Windows 17.0 (SPSS, Chicago, IL, USA). Home and office BP was given as the mean of two measurements. For home BP, the values from the last week of each period were used. The changes in response to head-up tilt were calculated as mean differences between supine values during minutes 3-5 and head-up tilt values during minutes 3-5, when the signal was most stable. The results are given as crude means with standard deviations or 95 % confidence intervals (CIs), unless stated otherwise. The normal distribution of variables was checked with the Shapiro-Wilk test before further analyses. The equality of variances among the study groups was tested with Levene's test for homogeneity of variances. Baseline values between the groups were compared using univariate analysis of variance, and changes between the groups with corresponding baseline value as a covariate. Analysis of variance for repeated measurements was used to test 1) interaction of time and group, 2) differences between groups, and 3) differences over time in continuous variables. Post hoc comparisons were adjusted with the Bonferroni correction. Adjustment for age was performed when appropriate. To compare within-group haemodynamic response to tilt at 0 and 12 weeks, paired samples t-test was utilized. Non-continuous variables were tested using Chi-square test. $P < 0.05$ was considered statistically significant.

Results

Study subjects and compliance

At baseline, there were no significant differences in sex distribution, body mass index, office BP, fasting plasma glucose and insulin, lipids, QUICKI insulin-sensitivity index⁽²⁵⁾, and estimated glomerular filtration rate⁽¹⁸⁾ between the groups (Table 1). The average QUICKI index values were clearly within the insulin-resistant range⁽²⁵⁾. Mean age was 4-5 years higher in the LTP25+PSe group than in the LTP5+PSe and placebo groups. In addition, in females the waist-to-height ratio⁽²⁶⁾ was higher in the LTP25+PSe than in the placebo group (Table 1). Body weight, and fasting plasma glucose, renin activity, aldosterone, potassium, sodium and potassium-to-sodium ratio remained virtually unchanged during the study (Table 2).

The subjects presented with the following diagnoses, but all of the clinical conditions listed below were stable and well-controlled or in remission: allergy (n=21), arthrosis (n=2), asthma (n=1), benign prostate hyperplasia (n=1), depression (n=11), epilepsy (n=1), fibromyalgia (n=1), gastro-

oesophageal reflux (n=1), Gilbert's syndrome (n=2), glaucoma (n=1), gout (n=4), hypothyroidism (n=8), paroxysmal atrial fibrillation (n=1), restless legs (n=1), rheumatoid arthritis (n=1), and systemic lupus erythematosus (n=1). The medications used by the subjects are tabulated in Supplemental Digital Content 1.

The mean (SD) duration of the intervention period was 84 (4), 83 (4), and 82 (5) days in the LTP5+PSe, LTP25+PSe, and placebo groups. Compliance was excellent, as only 1 subject (LTP5+PSe group) consumed <80 % of the test products. Although the subjects were instructed to maintain their normal lifestyle and dietary habits, some subjects reported minor modifications that are shown in Supplemental Digital Content 2.

Home BP measurements

Mean values of home BP measurements at weeks 0, 8 and 12 are presented in Table 3. Neither of the treatments influenced BP, as there were no significant group by time interactions in systolic and diastolic BP during the study. Home diastolic BP was lower in the LTP5+PSe than LTP25+PSe group ($p=0.009$), but neither of these groups differed from placebo. Home systolic and diastolic BP were slightly reduced in all groups from baseline to week 12 (Table 3).

Haemodynamic measurements in the laboratory

Haemodynamic *baseline data*, captured during the 15-min recording protocol, is shown in Figure 1. Analysis of variance for repeated measurements (age as covariate) showed a significant group by time interaction in radial systolic ($p=0.009$) and diastolic ($p=0.004$) BP and SVRI ($p=0.001$), but not in CI ($p=0.186$), during the 15-min protocol at baseline. Thus, functional haemodynamic regulation was not corresponding in all study groups, and the clearest difference was that the LTP5+PSe group showed lower supine, but not upright, DBP and SVRI than the placebo group.

Changes in the haemodynamic variables during the 15-min recording protocol from week 0 to week 12 are presented in Figure 2. Analysis of variance for repeated measurements (with age as covariate) showed no significant group by time interactions, or differences between the groups or within groups, in the changes in radial systolic and diastolic BP, SVRI or CI. Similarly, no BP lowering effects of LTP+PSe diets were observed at week 8 (data not shown).

The *cardiovascular response to head-up tilt* at baseline and after 12 weeks of intervention is shown in Figure 3. Within groups, there were no significant differences in the systolic BP or SVRI response to head-up tilt at week 0 and 12, but in the LTP5+PSe and placebo group the CI response

to head-up tilt showed a greater reduction at week 12 than at baseline ($p=0.008$ and $p=0.036$, respectively). In addition, the diastolic BP response to head-up tilt decreased in the LTP5+PSe group ($p=0.046$). The increase in SVRI during head-up tilt was lower in the LTP25+PSe than LTP5+PSe group both at baseline and week 12 ($p<0.01$), showing consistent functional differences in haemodynamics between these groups.

Plasma lipids and lipoproteins

The changes in plasma lipid concentrations from baseline to week 12 are presented in Table 4. There was a numerical but not statistically significant tendency towards lower plasma LDL cholesterol concentration after the intervention in the test groups ($p=0.066$). When the two test groups with similar daily doses of PSe were analysed together as one group, the test treatment decreased LDL cholesterol compared to placebo ($p=0.024$). Plasma total cholesterol, HDL cholesterol, and triglyceride concentrations were virtually unchanged in all study groups.

Discussion

At present the evidence about the putative beneficial effect of LTPs on blood pressure remains contradictory^(7,10-12). Therefore, we conducted for the first time a clinical trial utilizing detailed haemodynamic measurements to examine the possible BP-lowering efficacy of two doses of LTPs (5 vs. 25 mg daily) in a long-term 12-week intervention. Since simultaneous reduction in plasma lipids can be anticipated to provide additional cardiovascular benefits, both of the LTP-products were fortified with PSe (daily dose 2 grams). However, the present results showed that intake of Ile-Pro-Pro and Val-Pro-Pro with PSe in fermented milk product was without significant antihypertensive effect, but a borderline lipid-lowering effect was observed in 104 subjects with MetS. Of note, the results on BP were consistent in measurements performed both at home and in the laboratory. The study also uncovered a significant haemodynamic difference in the SVRI response to head-up tilt between the LTP5+PSe and LTP25+PSe groups both at baseline and after 12 weeks of intervention. Thus, these two groups showed persistent functional cardiovascular differences in the supine to upright regulation of peripheral vascular resistance.

Four meta-analyses including Asian and Caucasian subjects in 12 to 19 placebo-controlled trials, with interventions lasting from 4 to 21 weeks, and daily peptide doses ranging from 2 to 52.5 mg, reported that products containing bioactive peptides may reduce BP by about 4-5/2 (systolic/diastolic) mmHg⁽⁵⁻⁸⁾. Based on these previous studies, our 12-week intervention with doses of 5 and 25 mg of LTPs should have been sufficient for detecting a lowering effect on BP, if present. Of

note, LTPs have been suggested to show more pronounced blood pressure-lowering effect in hypertensive subjects than in prehypertensive subjects⁽⁷⁾. The present study population had a clearly elevated BP with a mean office BP at screening of 163/100 mmHg, and a mean home BP in the beginning of the intervention of 142/88 mmHg.

Corresponding to our results, Dutch intervention studies showed no significant decreases in BP after ingestion of 4.6-14 mg/d of Ile-Pro-Pro and Val-Pro-Pro for 4-8 weeks⁽¹⁰⁻¹²⁾. Furthermore, the BP-lowering effect of LTPs may be influenced by ethnic factors. In the meta-analysis by Cicero et al.⁽⁵⁾, the effect of LTP was more pronounced in Asian subjects (systolic BP -6.93 mmHg; diastolic BP -3.98 mmHg) than in European subjects (systolic BP -1.17 mmHg; diastolic BP -0.52), and was not related to age, baseline BP, dose of LTPs, or length of the treatment. One possibility is that differences in background diet may explain the variable responses to ingestion of LTPs.

Results from experimental studies have suggested that LTPs may have competitive ACE inhibiting activity⁽²⁷⁾ but this has not been confirmed in humans⁽²⁸⁾. ACE inhibitor drugs that are used in the treatment of cardiovascular disease have been reported to increase plasma renin activity, reduce plasma aldosterone concentration⁽²⁹⁻³¹⁾, and elevate plasma potassium concentration^(32,33). During the present study, there were no significant differences in plasma renin activity, plasma aldosterone and potassium concentrations, and plasma potassium-to-sodium ratio between the groups. These findings, together with the absence of changes in BP, indicate that the possible ACE inhibiting activity of the study products *in vivo* was not sufficiently strong to elicit detectable changes in the aforementioned variables.

Also other modes of antihypertensive action for LTPs, such as inhibition of the sympathetic nervous system, and an effect on cardiac output, have been suggested^(28,34). Intake of Val-Pro-Pro and Ile-Pro-Pro slightly improved variables of cardiac flow (stroke volume and stroke index) and contractility (acceleration index and velocity index), but was without effect on variables of fluid dynamics or vascular resistance in subjects with high-normal BP or mild hypertension⁽³⁴⁾. The present study showed no effect of LTPs on systemic vascular resistance and cardiac index, which is in agreement with the results reported by Cicero et al.⁽³⁴⁾. One previous study utilizing a tilt table test suggested that LTP intake may have a mild lowering effect on sympathetic activity, since plasma noradrenaline response to head-up tilt was reduced after 8 weeks of intervention, although no significant changes were seen in BP and heart rate⁽²⁸⁾. In our study, LTP treatment was without influence on radial BP either in supine position or during head-up tilt.

In the present investigation a simultaneous intake of LTP and PSe was used. In experimental studies, combined treatment with LTP and PSe was found to attenuate the elevation of blood pressure in spontaneously hypertensive rats⁽³⁵⁾. However, the antihypertensive effect of combined

LTP+PSe intake was less marked than the effect of LTP treatment alone⁽³⁵⁾, while PSe treatment alone did not affect BP⁽³⁶⁾. Further, plant sterols have even been reported to increase BP in salt-loaded stroke-prone spontaneously hypertensive rats⁽³⁷⁾. Based on these experimental results, it can be speculated whether the simultaneous administration of PSe attenuated the possible BP-lowering effect of LTPs in the present study, and whether an even higher dose of LTPs would have resulted in the lowering of BP. Nevertheless, in previous human studies, the simultaneous intake of LTPs and PSe for 10 weeks was shown to decrease systolic BP at home measurements but not in the office or during 24-h ambulatory recordings^(15,16). In addition, a recent study suggested an acute BP-lowering effect 8 h post-dose of fermented milk product containing 25 mg LTP and 2 g PSe in subjects with mild hypertension⁽³⁸⁾.

The present cholesterol-lowering effect of 2 g daily PSe intake remained modest when compared with a previous meta-analysis reporting a mean reduction of 0.31 mmol/l in plasma LDL cholesterol concentration⁽¹⁴⁾. Earlier, the efficacy of plant stanols and sterols has been studied in different food matrices including low-fat milk products⁽¹⁴⁾, and the beneficial effects have been confirmed in both normocholesterolemic and hypercholesterolemic subjects⁽³⁹⁾, as well as in type 2 diabetic patients⁽⁴⁰⁾. However, the efficacy of phytosterols in subjects with MetS is contradictory, since negative results have been reported after ingestion of plant sterol ester enriched milk, breakfast cereal and margarine^(41,42) while consumption of plant stanol ester yogurt drink have been shown to lower non-HDL cholesterol in MetS⁽⁴³⁾. In particular, reduced intestinal cholesterol absorption observed in MetS subjects may interfere with the plant sterols' mechanism of action and efficacy⁽⁴¹⁾. It should also be noted that seasonal variation of blood lipid levels in hypercholesterolemic subjects, with a characteristic peak of total and LDL cholesterol in fall/winter and trough in spring/summer⁽⁴⁴⁾, may have attenuated the cholesterol-lowering effect of the present study, half of which was conducted in fall. We observed a slight numeric increase in the mean values of total and LDL cholesterol of the placebo group.

In addition to hypertension and dyslipidemia, central obesity and insulin resistance are associated with the MetS⁽¹⁾. During the present intervention BP, body weight and plasma glucose remained unaltered in all study groups. We did not investigate the change in plasma insulin at close of the study. However, the absence of alterations in the above cardiometabolic risk factor suggests that the insulin sensitivity did not significantly change in the groups during the study.

The particular strength of this study is the randomized double-blind placebo-controlled design. In addition to home BP measurements, we utilized detailed cardiovascular reactivity test giving haemodynamic information in both supine position and during orthostatic challenge. Furthermore, we used two doses (5 and 25 mg) of LTPs and 12 week intervention. Compliance was excellent,

and according to the unchanged body weight the subjects were able to maintain their habitual lifestyle and dietary pattern during the intervention. Characteristically of studies examining the antihypertensive influences of various treatments, BP and SVRI were numerically lower after the 12-week follow-up also in the placebo group⁽⁴⁵⁾.

Our study has some limitations. There were differences between the groups in the demographic characteristics at baseline, in spite of the randomization protocol. Age was higher in the LTP25+PSe group than in the LTP5+PSe and placebo groups, and in female subjects waist-to-height ratio was higher in the LTP25+PSe than the placebo group. In addition, the results showed persistent functional differences in upright haemodynamics between the groups, since the increase in SVRI during orthostatic challenge remained lower in the LTP25+PSe than in the LTP5+PSe group throughout the study. A cross-over protocol, instead of the present parallel design, would have reduced possible confounding resulting from the divergent cardiovascular regulation in the study groups. However, differences in the control of peripheral vascular resistance during head-up tilt were difficult to anticipate, as there is very little previous information about dietary influences on upright cardiovascular regulation. As the present parallel study included two intervention groups and a control group during a relatively long-term period (12 weeks), a corresponding cross-over design with each subject receiving all treatments would have been more strenuous to carry out (36 weeks altogether), and this might also have reduced compliance. Finally, the consumption of the study products was monitored by use of the study diaries, and compliance was calculated from the registrations of the study subjects. In order to really ensure accurate intake of the study products, an intervention utilizing a laborious controlled feeding protocol should have been utilized⁽⁴⁶⁾.

In conclusion, this study showed no antihypertensive effect of intake of LTP and PSe in fermented milk product at home or in laboratory measurements, but a mild lipid-lowering effect in subjects with MetS.

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Conflicts of interest

TM and AMT are present, and SL and RK former employees of Valio Ltd. Also, we received fermented milk products, which were delivered to the participants in all three study groups, from commercial source Valio Ltd. Other authors have no conflict of interest.

Authorship

Formulating the research questions: MK, TM, AMT, HV, RK, IHP. Designing the study: MK, TM, AMT, HV, RK, IHP. Carrying it out: AJT, AT, SL, ON, IHP. Analysing the data: EJH, AJT, KN, IHP. Writing the article: EJH, AMT, HV, IHP.

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Table 1. Clinical and metabolic characteristics of the study subjects at baseline (n=104).

| | LTP5+PSe (n=36) | | LTP25+PSe (n=35) | | Placebo (n=33) | | ANOVA p value* |
|--------------------------------------------------|--------------------|-------|---------------------|-------|-------------------|-------|--------------------|
| | Mean | SD | Mean | SD | Mean | SD | |
| Age (years) | 49.5 | 6.9 | 54.3 ^{a,b} | 5.9 | 50.2 | 7.2 | 0.007 |
| Male/Female | 21/15 | | 22/13 | | 20/13 | | 0.927 [†] |
| Body mass index (kg/m ²) | 30.5 | 4.5 | 31.5 | 3.5 | 30.0 | 3.7 | 0.109 |
| Waist-to-height ratio [‡] | | | | | | | |
| Male | 0.60 | 0.06 | 0.61 | 0.04 | 0.59 | 0.05 | 0.322 |
| Female | 0.59 | 0.06 | 0.64 ^c | 0.06 | 0.58 | 0.04 | 0.044 |
| Office SBP (mmHg) | 159 | 12 | 168 | 20 | 162 | 15 | 0.229 |
| Office DBP (mmHg) | 97 | 8 | 102 | 10 | 100 | 10 | 0.100 |
| Haemoglobin (g/l) | 149 | 12 | 146 | 12 | 149 | 14 | 0.985 |
| <i>Fasting plasma</i> | | | | | | | |
| Total cholesterol (mmol/l) | 5.6 | 1.0 | 5.7 | 0.6 | 5.7 | 0.8 | 0.903 |
| HDL cholesterol (mmol/l) | 1.32 | 0.39 | 1.42 | 0.34 | 1.34 | 0.31 | 0.944 |
| LDL cholesterol (mmol/l) | 3.5 | 0.8 | 3.5 | 0.7 | 3.5 | 0.7 | 0.875 |
| Triglycerides (mmol/l) | 2.05 | 1.14 | 1.80 | 0.88 | 1.77 | 0.86 | 0.535 |
| Insulin (mU/l) | 10 | 7 | 8 | 4 | 9 | 3 | 0.406 |
| QUICKI index [§] | 0.147 | 0.015 | 0.158 | 0.040 | 0.150 | 0.011 | 0.473 |
| eGFR (ml/min/1.73 m ²) | 113 | 14 | 106 | 12 | 111 | 14 | 0.713 |

* Age as covariate.

^ap=0.009 compared with LTP5+PSe.

^bp=0.038 compared with placebo.

^cp=0.048 compared with placebo.

[†]Chi-square test.

[‡]Waist-to-height ratio⁽²⁶⁾.

[§]Insulin sensitivity QUICKI index⁽²⁵⁾.

^{||}RULE formula⁽¹⁸⁾.

Table 2. Body weight, and plasma glucose, electrolytes, renin, and aldosterone during the study.

| | LTP5+PSe (n=36) | | LTP25+PSe (n=35) | | Placebo (n=33) | | ANOVA p value* |
|----------------------------|--------------------|------|---------------------|------|-------------------|------|-------------------|
| | Mean | SD | Mean | SD | Mean | SD | |
| Weight | | | | | | | |
| Week 0 | 92.1 | 17.6 | 93.7 | 12.3 | 89.8 | 14.2 | 0.070 |
| Week 12 | 92.0 | 17.7 | 94.1 | 12.9 | 90.0 | 14.1 | 0.051 |
| Weight change in 12 weeks | -0.1 | 1.7 | 0.5 | 2.1 | 0.2 | 1.8 | 0.300 |
| <i>Plasma fasting</i> | | | | | | | |
| Glucose (mmol/l) | | | | | | | |
| Week 0 | 5.8 | 0.7 | 5.9 | 0.6 | 5.7 | 0.5 | 0.528 |
| Week 12 | 5.8 | 0.7 | 5.9 | 0.6 | 5.7 | 0.5 | 0.458 |
| Renin activity (µg/l/h) | | | | | | | |
| Week 0 | 0.58 | 0.48 | 0.69 | 0.68 | 0.90 | 0.74 | 0.111 |
| Week 12 | 0.80 | 0.89 | 0.79 | 0.84 | 1.03 | 0.91 | 0.470 |
| Aldosterone (pmol/l) | | | | | | | |
| Week 0 | 450 | 206 | 483 | 223 | 499 | 192 | 0.532 |
| Week 12 | 486 | 275 | 501 | 254 | 510 | 214 | 0.745 |
| Potassium (mmol/l) | | | | | | | |
| Week 0 | 4.0 | 0.3 | 4.1 | 0.2 | 4.0 | 0.3 | 0.748 |
| Week 12 | 3.9 | 0.3 | 3.9 | 0.2 | 3.9 | 0.3 | 0.396 |
| Sodium (mmol/l) | | | | | | | |
| Week 0 | 140 | 2 | 140 | 2 | 140 | 2 | 0.961 |
| Week 12 | 139 | 2 | 139 | 2 | 140 | 2 | 0.375 |
| Potassium:sodium ratio (%) | | | | | | | |
| Week 0 | 2.9 | 0.2 | 2.9 | 0.2 | 2.9 | 0.2 | 0.766 |
| Week 12 | 2.8 | 0.2 | 2.8 | 0.1 | 2.8 | 0.2 | 0.948 |

* Age as covariate.

Table 3. Home systolic and diastolic blood pressure during the intervention.

| | LTP5+PSe (n=36) | | LTP25+PSe (n=35) | | Placebo (n=32) | | ANOVA for repeated measures p [*] | | | ANOVA p [†] |
|------------------------------------------------------|--------------------|-------------|---------------------|-------------|-------------------|-------------|--------------------------------------------|---------------------------------|--------------------------------|----------------------|
| | Mean | SD (CI) | Mean | SD (CI) | Mean | SD (CI) | Group by time interaction | Between- group difference | Within- group difference | |
| SBP (mmHg) | | | | | | | | | | |
| 0 week | 139 | 11 | 147 | 17 | 139 | 14 | | | | |
| 8 week | 137 | 13 | 144 | 17 | 138 | 14 | | | | |
| 12 week | 138 | 13 | 145 | 17 | 137 | 15 | 0.746 | 0.055 | 0.170 | |
| SBP change in 12 weeks (mmHg)[‡] | -1.4 | (-3.9, 1.2) | -1.1 | (-4.7, 2.5) | -2.4 | (-5.3, 0.5) | | | | 0.274 |
| DBP (mmHg) | | | | | | | | | | |
| 0 week | 85 | 7 | 91 | 10 | 87 | 9 | | | | |
| 8 week | 84 | 8 | 89 | 9 | 85 | 8 | | | | |
| 12 week | 84 | 8 | 89 | 9 | 86 | 9 | 0.726 | 0.011 ^a | 0.299 | |
| DBP change in 12 weeks (mmHg)[‡] | -1.3 | (-3.0, 0.4) | -1.0 | (-3.1, 1.1) | -1.3 | (-3.1, 0.4) | | | | 0.256 |

*Age as covariate; [†]Age and baseline measure as covariates; [‡]SBP and DBP change in 12 weeks is expressed as means and 95 percent confidence intervals; ^aLTP25+PSe group differed from LTP5+PSe group (p=0.009) in post-hoc comparisons (Bonferroni corrections applied).

Table 4. Changes in plasma lipid concentrations during the study (n=104).

| | LTP5+PSe | | LTP25+PSe | | Placebo | | ANOVA p [*] |
|----------------------------|----------|--------------|-----------|-------------|---------|--------------|-------------------------|
| | (n=36) | | (n=35) | | (n=33) | | |
| | Mean | 95 % CI | Mean | 95 % CI | Mean | 95 % CI | |
| Total cholesterol (mmol/l) | -0.0 | -0.3, 0.2 | -0.1 | -0.3, 0.1 | +0.1 | -0.1, 0.2 | 0.465 |
| LDL cholesterol (mmol/l) | -0.1 | -0.3, 0.1 | -0.1 | -0.3, 0.0 | +0.1 | -0.1, 0.3 | 0.066 |
| HDL cholesterol (mmol/l) | -0.06 | -0.12, -0.00 | -0.05 | -0.12, 0.01 | -0.08 | -0.15, -0.02 | 0.716 |
| Triglycerides (mmol/l) | +0.02 | -0.32, 0.36 | +0.10 | -0.10, 0.29 | +0.11 | -0.20, 0.42 | 0.742 |

* Age and baseline value as covariates.

Figure legends

Figure 1. Radial systolic blood pressure (A), diastolic blood pressure (B), systemic vascular resistance index (C), and cardiac index (D) during haemodynamic recordings performed at baseline. All subjects underwent the 15-min recordings during which the 5-min head-up tilt was performed between 5 and 10 min. Mean and SEM depicted.

Figure 2. Changes in radial systolic blood pressure (A), diastolic blood pressure (B), systemic vascular resistance index (C), and cardiac index (D) during the 15-min recording protocol as calculated from the 1st recording (at 0 weeks) to the 3rd recording (after 12 weeks of intervention). The 5-min head-up tilt was performed between 5 and 10 min. Mean and SEM depicted.

Figure 3. Haemodynamic responses to upright posture in radial systolic blood pressure (A), diastolic blood pressure (B), systemic vascular resistance index (C), and cardiac index (D) at baseline and after 12 weeks of intervention. Within groups, the haemodynamic responses at weeks 0 and 12 did not differ, apart from the accentuated CI decline in the LTP5+PSe and placebo group at week 12 ($p=0.008$ and $p=0.036$, respectively), and the diastolic BP decline in the LTP5+PSe group ($p=0.046$). Paired samples t-test applied in within group comparisons, ANCOVA with age as covariate applied in between group comparisons, with Bonferroni corrections in post hoc comparisons, mean and SEM depicted, * $p<0.05$, ** $p<0.01$.

List of Supplemental Digital Content

1. Hautaniemi Supplemental Digital Content 1.pdf
2. Hautaniemi Supplemental Digital Content 2.pdf

Figure 1.

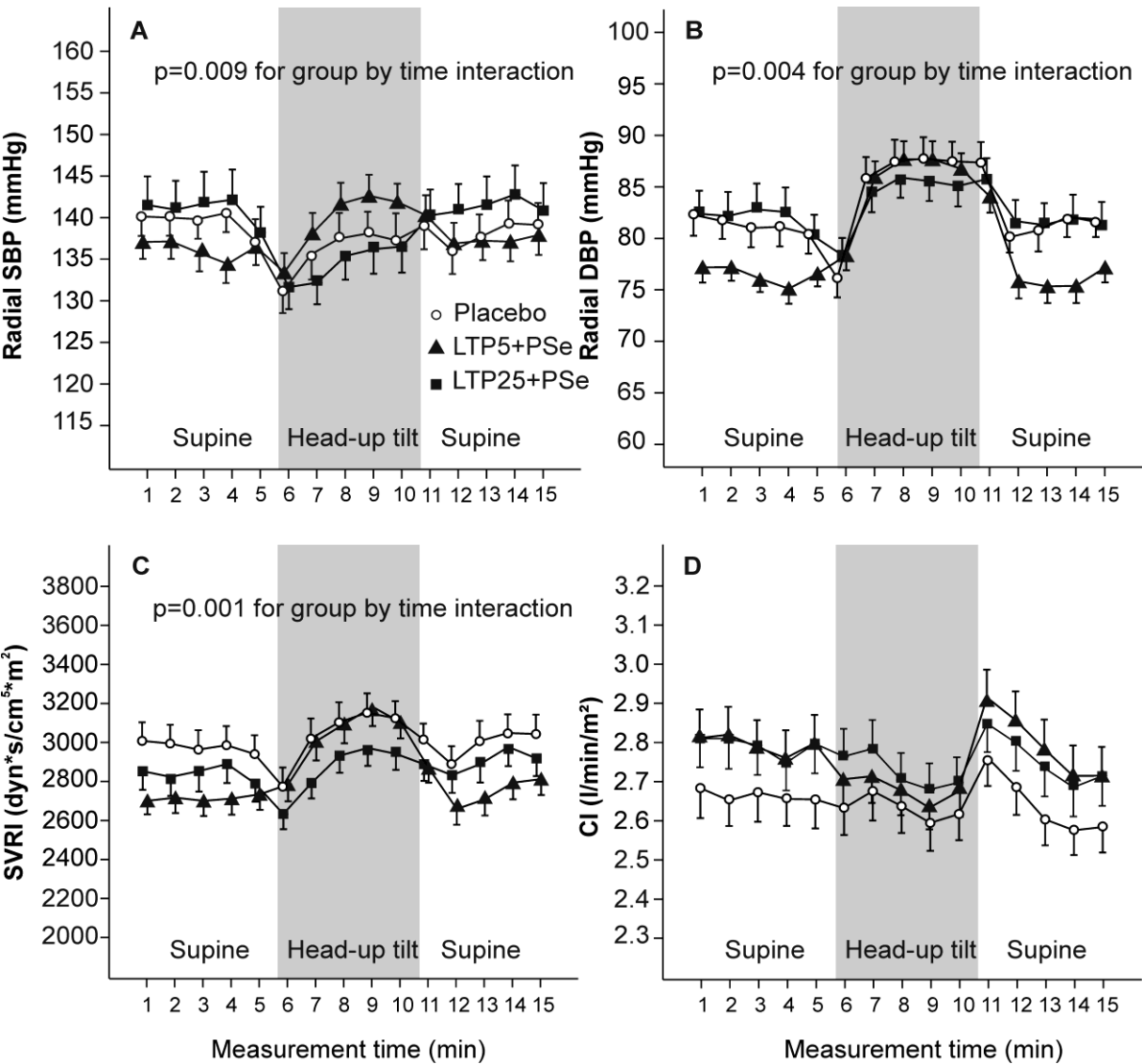


Figure 2.

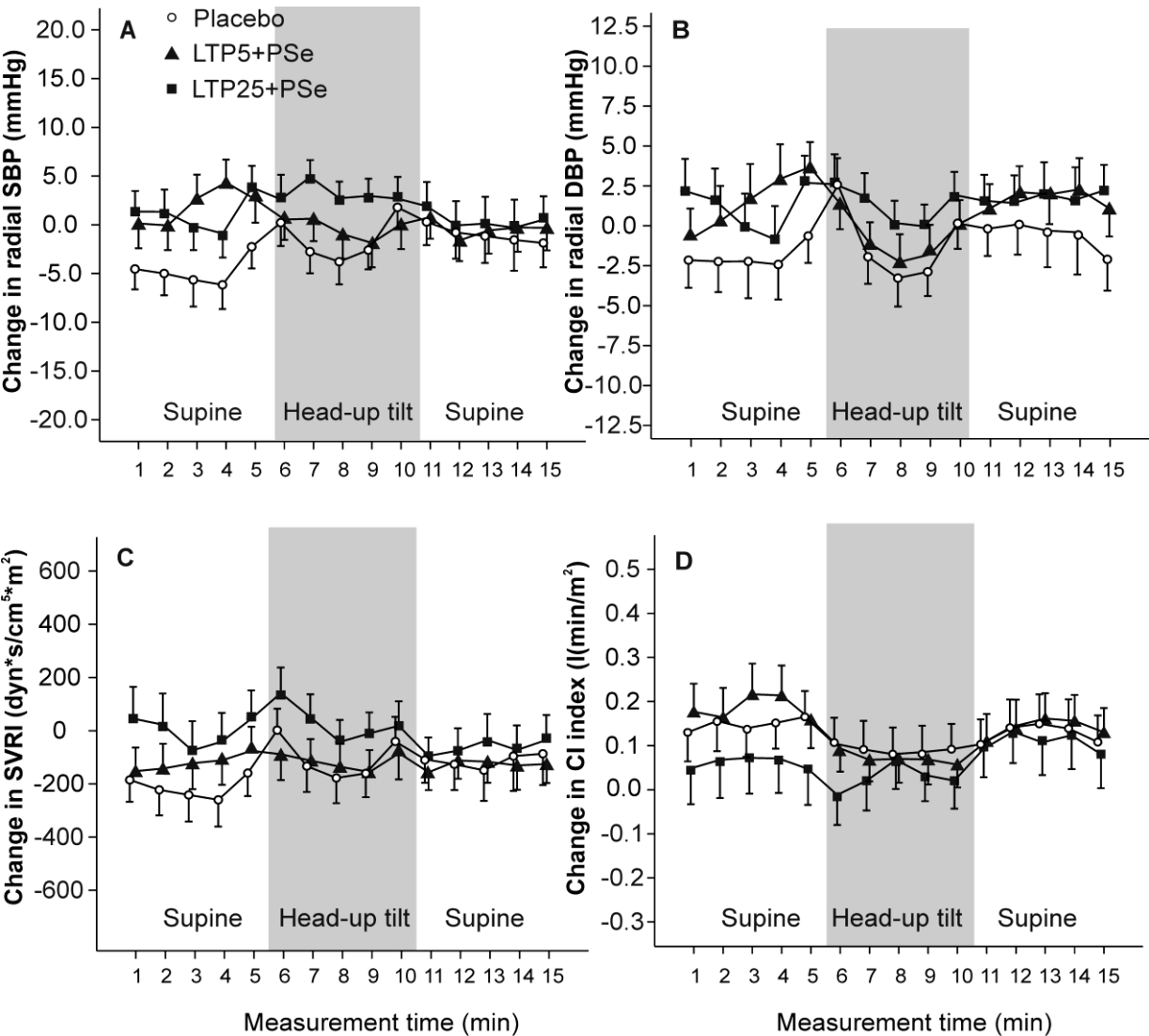
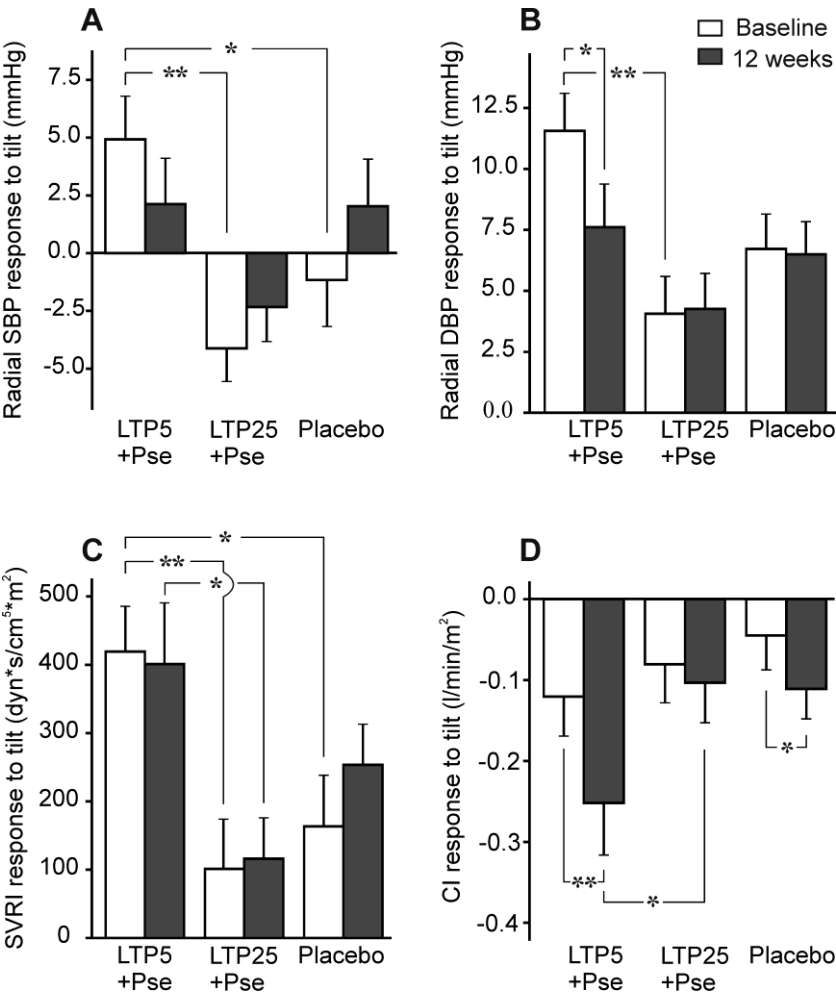


Figure 3.



PUBLICATION

II

Daily Liquorice Consumption for Two Weeks Increases Augmentation Index and Central Systolic and Diastolic Blood Pressure

Leskinen MH, Hautaniemi EJ, Tahvanainen AM, Koskela JK, Päällysaho M, Tikkakoski AJ, Kähönen M, Kööbi T, Niemelä O, Mustonen J, Pörsti IH

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Daily Liquorice Consumption for Two Weeks Increases Augmentation Index and Central Systolic and Diastolic Blood Pressure

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Abstract

Background: Liquorice ingestion often elevates blood pressure, but the detailed haemodynamic alterations are unknown. We studied haemodynamic changes induced by liquorice consumption in 20 subjects versus 30 controls with average blood pressures of 120/68 and 116/64 mmHg, respectively.

Methods: Haemodynamic variables were measured in supine position before and after two weeks of liquorice consumption (daily glycyrrhizin dose 290–370 mg) with tonometric recording of radial blood pressure, pulse wave analysis, and whole-body impedance cardiography. Thirty age-matched healthy subjects maintaining their normal diet were studied as controls.

Results: Two weeks of liquorice ingestion elevated peripheral and central systolic and diastolic blood pressure (by 7/4 and 8/4 mmHg, 95% confidence intervals [CI] 2–11/1–8 and 3–13/1–8, respectively, $P < 0.05$), and increased extracellular volume by 0.5 litres ($P < 0.05$ versus controls). Also augmentation index adjusted to heart rate 75/min (from 7% to 11%, 95% CI for change 0.3–7.5, $P < 0.05$) and aortic pulse pressure (by 4 mmHg, 95% CI 1–7, $P < 0.05$) were elevated indicating increased wave reflection from the periphery. In contrast, peripheral (–3/–0.3 mmHg) and central blood pressure (–2/–0.5 mmHg), aortic pulse pressure (–1 mmHg), and augmentation index adjusted to heart rate 75/min (from 9% to 7%) decreased numerically but not statistically significantly without changes in extracellular volume in the control group. Heart rate, systemic vascular resistance, cardiac output, and pulse wave velocity did not differ between the groups.

Conclusions: Two weeks of daily liquorice consumption increased extracellular volume, amplified pressure wave reflection from the periphery, and elevated central systolic and diastolic blood pressure.

Trial Registration: EU Clinical Trials Register EudraCT 2006-002065-39 ClinicalTrials.gov NCT01742702

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Introduction

During recent years there has been a growing research interest concerning the role of mineralocorticoids and the mineralocorticoid receptor (MR) in hypertension [1]. MRs are found in both classical aldosterone target tissues (kidneys, colon and salivary glands), but also in non-classical target tissues like the heart, vascular wall, and the central nervous system [2].

Both aldosterone and cortisol can bind to the MR with comparable affinity *in vitro* [3,4]. However, normally only aldosterone acts as the physiologic agonist of the MR [3,4], although the circulatory concentration of cortisol is 200 to 1000-

fold higher than that of aldosterone. This paradox was solved with the discovery of the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2), which protects the MR by degrading biologically active cortisol to inactive cortisone [3,4]. Liquorice contains glycyrrhizinic acid, which in the bowel is metabolized to glycyrrhetic acid [5], which in turn inhibits 11 β -HSD2 and enhances the action of cortisol on the MR [6]. Therefore, the liquorice-induced elevation of blood pressure is mediated via the MR [7].

In the kidneys the MR is also protected from cortisol by 11 β -HSD2 [3,4]. Excessive renal MR activation by cortisol promotes

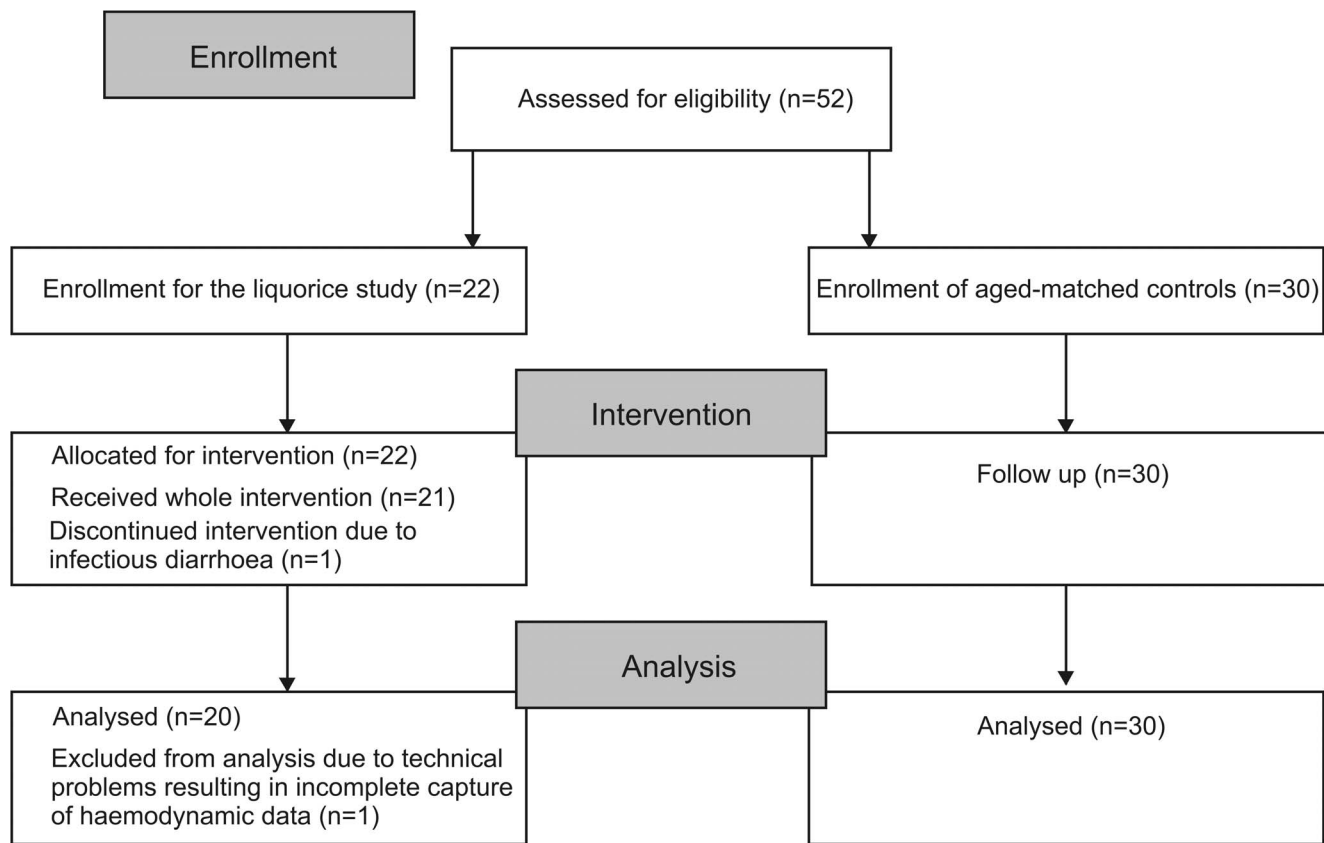


Figure 1. Flow diagram of the study.
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reabsorption of sodium from renal distal tubule and increases excretion of potassium and hydrogen into the urine. This leads to sodium retention, increased extracellular volume, hypertension, hypokalemia and metabolic alkalosis. This condition is called pseudohyperaldosteronism due to low renin and low aldosterone concentrations in plasma, as described in 1968 by Conn [8].

The enzyme 11β -HSD2 is also present in the human vascular wall in both smooth muscle cells and endothelial cells, where it can influence vascular tone by modulating the access of glucocorticoids to the MR [9]. Interestingly, MR blockade can have blood pressure-independent beneficial effects on arterial stiffness [10]. In the heart the presence of 11β -HSD2 is somewhat controversial [1], but it has also been suggested that this enzyme is co-expressed with the MR in the human cardiac tissue [11].

The sensitivity of human subjects to the liquorice-induced elevation of blood pressure varies individually and patients with hypertension show higher sensitivity [12]. Based on the mechanisms described above, the elevation of blood pressure could result from increased extracellular volume caused by sodium retention, enhanced peripheral arterial resistance due to vasoconstriction, increased large arterial stiffness via MR activation, or changes in cardiac function. Since the detailed hemodynamic effects remain unknown, we examined the haemodynamic changes induced by liquorice ingestion in healthy volunteers.

Materials and Methods

The original study protocol (in Finnish) and English summary of study protocol, and supporting Consort checklist, and data are

available as supporting information; see Protocol S1, Protocol S2, Checklist S1 and Data S1.

Ethics statement

All participants signed informed study consent. This study was approved by the Ethics Committee of the Tampere University Hospital (study code R07053M), complies with the principles outlined in the Declaration of Helsinki, and is registered in the database of clinical trials (ClinicalTrials.gov, ID: NCT01742702).

The haemodynamic recording protocol was registered in the EU Clinical Trials Register (EudraCT-number 2006-002065-39), and the study was approved by the Ethics Committee of Tampere University Hospital and by the Finnish Medicines Agency (code numbers R06086M for controls and R07053M for liquorice diet). The ongoing haemodynamic study was also registered in the database of clinical trials (ClinicalTrials.gov, ID: NCT01742702). The authors confirm that all ongoing and related trials for this intervention are registered.

Study design and participants

This was an open-label study, and all participants were recorded before and after the 2-week intervention (liquorice ingestion) or the 1–3 week follow-up (normal diet). Experts from a Finnish sweet manufacturer (www.fazer.com) advised us that a product that would have authentic liquorice taste in the absence of glycyrrhizin could not be prepared, and therefore a double-blind crossover study design was not possible. The study subjects were recruited through noticeboard or email announcements from the

personnel and students of the University of Tampere and Tampere University Hospital. In addition, professionals in local occupational health care units were informed that their patients could participate in an ongoing study on haemodynamics that included medical inspection and routine laboratory analyses (Figure 1). The data were collected from June 2006 to June 2011 at the Department of Clinical Physiology, Tampere University Hospital, Finland.

The *liquorice group* initially consisted of 22 normotensive volunteers, but one subject discontinued the study due to infectious diarrhoea. In addition, capture of haemodynamic data was incomplete in one subject, who was excluded from final analyses. The final *liquorice group* consisted of 20 subjects (12 women and 8 men, aged 33.6 ± 2 years), who ingested liquorice daily for two weeks. Preceding the study, the subjects were instructed to abstain from liquorice-containing products for 3 weeks. The participants were allowed to choose between two commercial products (Halva liquoriceTM, Kouvola liquoriceTM) so that the estimated daily dose of glycyrrhizin was 290–370 mg. If the daily glycyrrhizin dose exceeds 400 mg, the risk of adverse events would be increased [13]. The glycyrrhizin content in these products was determined in 1993 [14], and according to the manufacturers, the preparation processes have not changed. Ethical approval allowed a liquorice intervention for a maximum period of 4 weeks. However, to ensure compliance the study was carried out using a 2-week-long liquorice diet. The dose and duration of the liquorice diet were based on experience gained by two members of the research group, who committed themselves to daily liquorice ingestion.

Normal diet group. To distinguish the possible effect of repeated haemodynamic measurements from the cardiovascular effects of liquorice consumption we recruited an age-matched group of 30 people. These subjects were not aware of serving as a control group for the liquorice study and during the measurements they were advised to maintain their normal diet. None in the normal diet group reported to consume liquorice containing products daily or weekly.

Exclusion criteria were office blood pressure over 140/90 mmHg, any cardiovascular disease with regular medication, or pregnancy. The participants were advised to inform the study group immediately if they gained weight over 4 kilograms, observed oedema in the lower extremities or encountered other problems during liquorice ingestion. Such problems were not reported, and 21 subjects maintained the liquorice diet for 2 weeks.

The suitability of all subjects for the study was confirmed by medical and laboratory examinations. The lifestyle habits, medical history and family history were recorded. In the liquorice group one subject was on regular medication for asthma (160 µg budesonide +4.5 µg formoterol twice daily), one for depression (escitalopram 5 mg once daily), one on postmenopausal oestrogen replacement therapy, and five female subjects used oral contraceptives. In the normal diet group 5 female subjects used oral contraceptives and 3 had hormonal intrauterine devices. There were 4 current smokers in the liquorice diet group, and 3 current and 4 previous smokers in the normal diet group.

Laboratory analyses

Standard electrocardiograms with 12 leads were taken with MAC5000 (GE Healthcare, Chalfont St. Giles, UK), and the recordings were normal in all participants. Before the study, blood and urine samples were collected after 12 hours of fasting, while in the normal diet group laboratory samples were not obtained from one subject. Plasma sodium, potassium, creatinine, glucose, cholesterol lipoproteins were analysed using Cobas Integra 700/

800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland), and blood haemoglobin by ADVIA 120 or 2120 (Bayer Health Care, Tarrytown, NY, USA). Plasma aldosterone concentration was determined using radioimmunoassay (Aldosterone RIA Test DSL-8600, Diagnostics Systems Laboratories Inc, Webster, TX, USA).

Blood pressure monitoring and pulse wave analysis

Blood pressure and pulse wave form were continuously recorded for 5 minutes from the left radial artery by a tonometric sensor (Colin BP-508T, Colin Medical Instruments Corp., USA). The tonometric recordings were calibrated twice during every 5-minute period using automated contralateral brachial blood pressure measurements. The SphygmoCor PWMx pulse wave monitoring system (Atcor Medical, Australia) was used to derive aortic blood pressure by previously reported generalized transfer function [15]. Heart rate, aortic pulse pressure (PP), augmentation index (AIx) (augmentation pressure/PP*100), and AIx adjusted to heart rate 75/min (AIx@75) were also determined. In addition, blood pressure was monitored from the left 3rd finger (Finometer^R model 1, Finapres Medical Systems, Amsterdam, the Netherlands).

Whole-body impedance cardiography

Whole-body impedance cardiography (CircMon^R, JR Medical Ltd., Tallinn, Estonia), based on the changes in electrical impedance of the body during cardiac cycle, was used to determine beat-to-beat heart rate, stroke volume, cardiac index, extracellular volume, and pulse wave velocity (PWV) [16]. Systemic vascular resistance index was calculated from radial blood pressure and cardiac index [17]. With the Circmon^R whole-body impedance cardiography method, i) the measurement of stroke volume shows good correlation with 3-dimensional echocardiography recordings [18], ii) the measurement of cardiac output shows good agreement with the thermodilution method [16], and the reproducibility and repeatability of both cardiac output and PWV measurements is good [16,18].

Haemodynamic measurements

Before recordings of pulse wave analysis and whole-body impedance cardiography, brachial systolic and diastolic blood pressure (SBP and DBP, respectively) was manually measured in the supine position using a standard sphygmomanometer, and Korotkoff sounds at phase V were used for estimating DBP.

The haemodynamic measurements were performed in a temperature-controlled laboratory before and after 2 weeks of liquorice ingestion. In the normal diet group the interval between the recordings was 1–3 weeks. The subjects were in the supine position on the examination table with electrodes on the surface of thorax and distally on the medial surface of extremities, tonometric sensor for pulse wave analysis on the left radial artery, and a cuff for the calibration of radial blood pressure in the right brachium [17]. Plethysmographic blood pressure sensor was placed on the 3rd left finger. The extended left upper limb was held at the level of the heart. Hemodynamic variables were captured continuously for 5 minutes, and average values from the whole period were used in the analyses. We have previously shown the good repeatability and reproducibility of the measurements [19].

Statistical analyses

The primary endpoints were significant changes in BP and other haemodynamic variables during the study. Secondary endpoints were changes in weight and plasma aldosterone and potassium concentrations. The power calculations were based on

expected changes in SBP according to the primary aim of the study. The required minimum sample size was 17 experimental and 26 control subjects to detect a 9 mmHg difference in the change in SBP from baseline (alpha level 0.05, 80% power, standard deviation (SD) 10 for variability, power analysis method for two-sample t-test). Normally distributed data was expressed as mean \pm SD or 95% confidence interval (95% CI). The results are given as mean \pm SD unless otherwise stated. General linear model of repeated measurements and t-test for paired samples were used to compare differences between measurements and groups. A chi-square test was used to compare gender distribution in groups, and Pearson's or Spearman's correlations (r) were calculated, as appropriate. $P < 0.05$ was considered significant. Statistical analyses were made using SPSS 17.0 (SPSS Inc., Chicago, Ill., USA).

Results

Demographic data and baseline laboratory values are presented in Table 1. At baseline there were no significant differences in any of the measured variables between the groups. Extracellular water and weight did not change during the normal diet, but after two weeks of liquorice diet extracellular water volume increased by 0.5 (± 1.4) litres and body weight by 1.5 kg (± 1.5) ($P = 0.04$ and $P = 0.002$, between the groups, respectively) (Table 2).

In manual measurements, brachial SBP/DBP increased by 2/3 ($\pm 7/5$) mmHg in the liquorice diet group ($P = 0.310$ for SBP and $P = 0.031$ for DBP) and decreased by $-3/-4$ ($\pm 5/6$) mmHg in the normal diet group ($P = 0.002$ for SBP and $P = 0.004$ for DBP) (Table 2) during the study. These moderate changes in SBP and DBP were significantly different between the groups ($P = 0.007$ for SBP and $P = 0.001$ for DBP). Liquorice ingestion significantly decreased plasma aldosterone ($P < 0.001$) and potassium concentrations ($P = 0.02$) (Table 2).

In tonometric measurements, the mean change (95% CI) between the visits in radial SBP/DBP in the liquorice group was 7/4 (2-11/1-8) mmHg (within group P -value < 0.001 for SBP and $P = 0.016$ for DBP) and in the normal diet group $-3/-0.3$ ($-5-0.4/-3-2$) mmHg ($P = 0.346$ for SBP and $P = 0.664$ for DBP). The differences between the groups were significant ($P < 0.001$ for

radial SBP and $P = 0.02$ for radial DBP) (Figure 2). The manual measurements of brachial SBP and DBP correlated well with tonometric radial measurements (during visit 2, $r = 0.85$ for SBP, $P < 0.001$; $r = 0.81$ for DBP, $P < 0.001$).

Comparable changes in blood pressure were also observed in finger measurements, as the mean change (95% CI) between the visits in finger SBP/DBP in the liquorice group was 5/4 (1-9/-1-8) mmHg (within group $P = 0.010$ for SBP and $P = 0.102$ for DBP), and in the normal diet group $-2/-2$ ($-7-3/-5-2$) mmHg ($P = 0.402$ for SBP and $P = 0.352$ for DBP). The mean change in finger SBP differed between the groups ($P = 0.030$), while the change in DBP did not reach statistical significance ($P = 0.065$). The manual measurements of brachial blood pressure and the plethysmographic measurements of finger blood pressure confirmed the significant effect of the liquorice intervention on blood pressure.

Aortic SBP/DBP increased in the liquorice group (8/4 mmHg, 95% CI 3-13/1-8, $P = 0.001$ for SBP and $P = 0.012$ for DBP), while no significant change was observed in the normal diet group ($-2/-0.5$ mmHg, 95% CI $-4-1/-3-2$, $P = 0.367$ for SBP and $P = 0.635$ for DBP). The mean change between the visits in aortic SBP/DBP differed between the groups ($P < 0.001$ for aortic SBP and $P = 0.02$ for aortic DBP, Figure 2). No correlations were found between the change in weight and the changes in radial or aortic blood pressures, and the change in extracellular water did not correlate with the changes in radial or aortic blood pressures, either (data not shown).

In the liquorice group aortic PP, AIx, and AIx@75 increased significantly: aortic PP was elevated by 4 (1-7) mmHg ($P = 0.01$, Figure 3), AIx increased from 13.7% to 18.7% (95% CI for change 1.3-8.9, $P = 0.012$), and AIx@75 increased from 7.3% to 11.2% (95% CI for change 0.3-7.5, $P = 0.031$, Figure 3). In the normal diet group aortic PP, AIx and AIx@75 did not significantly change between the visits: the difference in aortic PP was -1 (95% CI $-3-0.2$) mmHg ($P = 0.281$), AIx values were 13.3% vs. 12.4% (95% CI for change $-2.9-1.1$, $P = 0.355$), and AIx@75 values were 8.6% vs. 6.9% (95% CI for change $-3.4-0.2$, $P = 0.669$). The differences between the groups were significant for aortic PP,

Table 1. Demographic data and laboratory values at baseline.

| | Liquorice diet n = 20 | Normal diet n = 29-30* | P value |
|--------------------------------------|-----------------------|------------------------|---------|
| Sex (F/M) | 12/8 | 17/13 | 0.53 |
| Age (years) | 33.5 \pm 7.9 | 33.8 \pm 8.1 | 0.91 |
| Height (cm) | 174 \pm 9 | 174 \pm 8 | 0.82 |
| Body mass index (kg/m ²) | 23.3 \pm 1.9 | 23.0 \pm 2.7 | 0.69 |
| Haemoglobin (g/l) | 140 \pm 8 | 143 \pm 15 | 0.40 |
| Fasting plasma | | | |
| Cholesterol (mmol/l) | 4.5 \pm 0.7 | 4.4 \pm 0.8 | 0.48 |
| Triglycerides (mmol/l) | 0.8 \pm 0.3 | 0.9 \pm 0.5 | 0.36 |
| High density lipoprotein (mmol/l) | 1.8 \pm 0.4 | 1.7 \pm 0.4 | 0.37 |
| Low density lipoprotein (mmol/l) | 2.3 \pm 0.6 | 2.2 \pm 0.7 | 0.59 |
| Glucose (mmol/l) | 5.2 \pm 0.4 | 5.0 \pm 0.4 | 0.13 |
| Creatinine (μ mol/l) | 79 \pm 15 | 76 \pm 11 | 0.39 |
| Sodium (mmol/l) | 140 \pm 1.2 | 140 \pm 2.0 | 0.84 |
| Potassium (mmol/l) | 3.9 \pm 0.3 | 3.9 \pm 0.2 | 0.73 |

All other values are mean \pm SD except for sex, which shows the number of subjects.

*Blood samples for fasting plasma values were not obtained from one subject.

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Table 2. Changes in weight, extracellular water, brachial office systolic and diastolic blood pressure, plasma aldosterone and plasma potassium after two weeks of liquorice ingestion.

| | Liquorice diet n = 20 | Normal diet n = 29–30* | P value |
|----------------------------------------|-----------------------|------------------------|---------|
| Weight (kg) | | | |
| Before liquorice ingestion | 70±9 | 71±9 | 0.73 |
| After liquorice ingestion | 72±9 [‡] | 71±8 | 0.87 |
| Extracellular water (l) | | | |
| Before liquorice ingestion | 12.5±1.4 | 12.7±1.2 | 0.47 |
| Change after liquorice ingestion | 0.5±1.4 | −0.1±0.5 | 0.04 |
| Office systolic blood pressure (mmHg) | | | |
| Before liquorice ingestion | 120±8 | 116±14 | 0.24 |
| Change after liquorice ingestion | 1.7±7.0 | −3.1±4.9 | 0.01 |
| Office diastolic blood pressure (mmHg) | | | |
| Before liquorice ingestion | 71±8 | 72±11 | 0.76 |
| Change after liquorice ingestion | 2.8±5.0 | −3.5±6.2 | 0.001 |
| Aldosterone (pmol/l) | | | |
| Before liquorice ingestion | 522±277 | 840±1202 [#] | 0.25 |
| After liquorice ingestion | 197±75 [‡] | not determined | - |
| Potassium (mmol/l) | | | |
| Before liquorice ingestion | 3.9±0.3 | 3.9±0.2 | 0.73 |
| After liquorice ingestion | 3.7±0.3 [‡] | not determined | - |

All values are mean ± SD.

*Blood samples for fasting plasma values were not obtained from one subject.

[‡]*P* = 0.02 and [‡]*P* < 0.001 versus the corresponding value before liquorice diet.

[#]Two normotensive and normokalemic subjects showed divergent serum aldosterone concentrations (6138 and 3720 pmol/l). If these subjects were excluded from the analysis, mean serum aldosterone concentration in the normal diet group was 548±340 pmol/l (*p* = 0.78 between groups).

doi:10.1371/journal.pone.0105607.t002

AIx, and AIx@75 (*P* = 0.001, *P* = 0.003, and *P* = 0.003, respectively).

PWV, an acknowledged marker of arterial stiffness, did not significantly change in either group (*P* = 0.191 and *P* = 0.481, liquorice and normal diet groups, respectively), and did not significantly differ between the groups (*P* = 0.19, Figure 3). The small changes in PWV, albeit statistically insignificant, correlated with changes in radial and aortic SBP and DBP (*r* = 0.40–0.45 for all, *P* values ranging 0.001–0.005). There were no significant differences in heart rate, cardiac index, and systemic vascular resistance index in the measurements within the groups (*P* > 0.05 for all), or between the groups (*P* = 0.55, 0.91 and 0.18 for heart rate, cardiac index and systemic vascular resistance index, respectively, Figure 4).

Discussion

Although liquorice ingestion is a known cause for diet-induced hypertension, the detailed haemodynamic changes underlying the elevation of blood pressure have not been reported after regular liquorice exposure. In this study we captured haemodynamic data before and after two weeks of liquorice ingestion, and found that the diet elevated both peripheral and central blood pressure and increased extracellular volume. The findings that central PP, AIx, and AIx@75 were also increased following the liquorice diet indicate that pressure wave reflection from the peripheral circulation was enhanced. In contrast, PWV, an acknowledged marker of arterial stiffness, did not significantly change, and neither did cardiac output nor systemic vascular resistance. Although blood pressure has been defined as the product (not

sum) of cardiac output and peripheral vascular resistance [20], moderate changes in the level of blood pressure are thus possible in the absence of statistically significant changes in these two principal variables. Taken together, the liquorice-induced elevation of blood pressure was associated with increased extracellular volume and enhanced pressure wave reflection from the peripheral arterial tree.

The recordings of tonometric radial and plethysmographic finger blood pressure, pulse wave analysis, and whole-body impedance cardiography were automated procedures devoid of subjective components, which can be argued to increase the reliability of the recordings. The study groups were homogenous so that different haemodynamic profiles associated e.g. with excess body weight and ageing could be avoided [21]. Although individual differences exist in the metabolism of glycyrrhizin to glycyrrhetic acid in the bowel and in the absorption of the latter, the glycyrrhizin content (w/w%) of the consumed product is the most important determinant of the clinical response to liquorice [22]. In the present study, increased extracellular volume and decreased plasma aldosterone and potassium levels documented the compliance of the participants as obvious consequences of the MR activation. Of note, subjects in the normal diet group were unaware that they served as controls for the liquorice diet, so they could not subconsciously change their dietary habits regarding liquorice containing products.

The type 1 and type 2 isoforms of 11β-HSDs regulate local cortisol metabolism. The 11β-HSD2 is mainly expressed in aldosterone target tissues [23], while both 11β-HSD isoenzymes are present in the vascular wall [24]. Arteries are thus a target tissue for mineralocorticoids and glucocorticoids, which via the

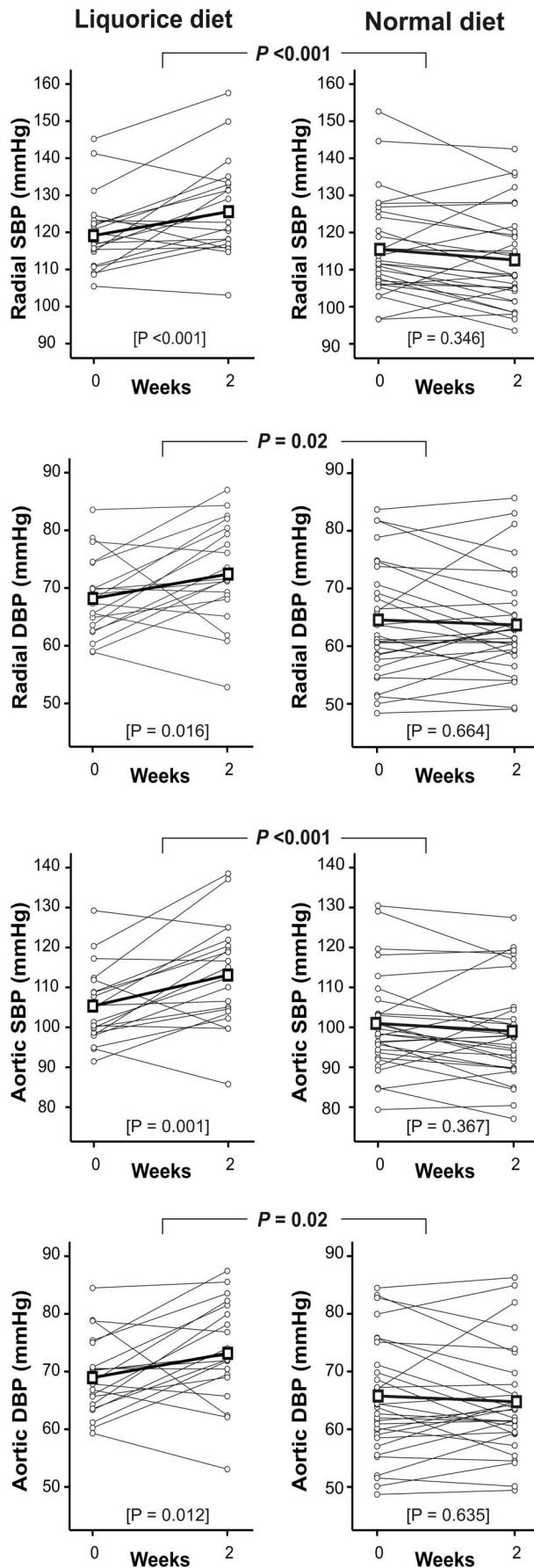


Figure 2. Radial and aortic blood pressure, liquorice diet versus normal diet. Grey lines represent each individual, thick black line represents mean values of each variable. SBP, systolic blood pressure; DBP, diastolic blood pressure; P values between graphs are for the difference in the change of each variable between liquorice diet versus normal diet, P values in brackets are for the change within each group, $n=20$ and 30 in the liquorice and normal diet groups, respectively.

doi:10.1371/journal.pone.0105607.g002

MR and GR can potentiate vascular responses to pressor hormones like α -adrenoceptor agonists and angiotensin II [25]. Of note, the effects of liquorice ingestion on 11β -HSD2, plasma electrolytes, and the renin-angiotensin-aldosterone axis can be long-lasting, since abnormalities in plasma electrolyte levels and urinary cortisol excretion may persist for 1–2 weeks after cessation of liquorice ingestion [26]. Moreover, after prolonged regular liquorice ingestion, the normalization of suppressed plasma renin and aldosterone values may take up to 2–4 months [27].

Recent studies have suggested that central blood pressure and PP are superior to brachial blood pressure in evaluating the risk of future cardiovascular events [28–30]. In a meta-analysis the predictive value of central AIX on cardiovascular events and all-cause mortality was even suggested to be independent of the level of blood pressure and heart rate [31]. In the present study, AIX was increased after liquorice diet, but PWV, an acknowledged measure of arterial stiffness, was not changed. Wave reflection occurs predominantly at the origin of the terminations of low resistance arteries into high-resistance arterioles, and stiffening of large arteries is known to result in earlier return of the reflected wave and higher AIX [32]. When large arterial stiffening results in earlier return of the reflected waves from the periphery to the heart, this process increases aortic SBP but reduces aortic DBP [32,33]. Thus, the present finding of increased aortic DBP following the liquorice diet agrees with the view that changes in arterial stiffness were not the cause for the alterations in central BP. Importantly, the level of AIX is not only determined by arterial stiffness, but also by systemic vascular resistance and other factors including sex, height and heart rate; actually the detailed determinants of AIX are still a matter of debate [32–34]. It seems possible that increased extracellular volume might also have an influence on the magnitude of AIX. Finally, it should be noted that arterial stiffness depends greatly on the prevailing blood pressure that is distending the blood vessels [35]. Although the present small changes in PWV were not statistically significant, they correlated with parallel changes in radial and aortic SBP and DBP. The present 2-week observation period was too short for structural changes to take place that would increase arterial stiffness, but such changes can be anticipated to result from longer periods of elevated blood pressure [36].

In the present study, the elevation of SBP and DBP caused by liquorice (on average 7/4 mmHg) was close to the efficacy of antihypertensive monotherapy in meta-analysis on pharmacotherapy of hypertension (average reduction 9/6 mmHg) [37]. Liquorice ingestion should be considered when taking the history of a hypertensive patient, but subjects may not always be aware that they have consumed liquorice containing products. In addition to sweets, many other food supplies contain glycyrrhizin as a sweetener or flavouring agent, including confectionery, beverages, alcohol drinks, cough mixtures, herbal medicines, health foods, and chewing tobacco. Moreover, dozens of medicinal preparations contain liquorice or liquorice derivatives [38]. According to the Finnish customs (www.tulli.fi), the amount of imported liquorice root extract was 686.000 kg during a period of 12 months (statistics from May 2013), and this would correspond to a daily

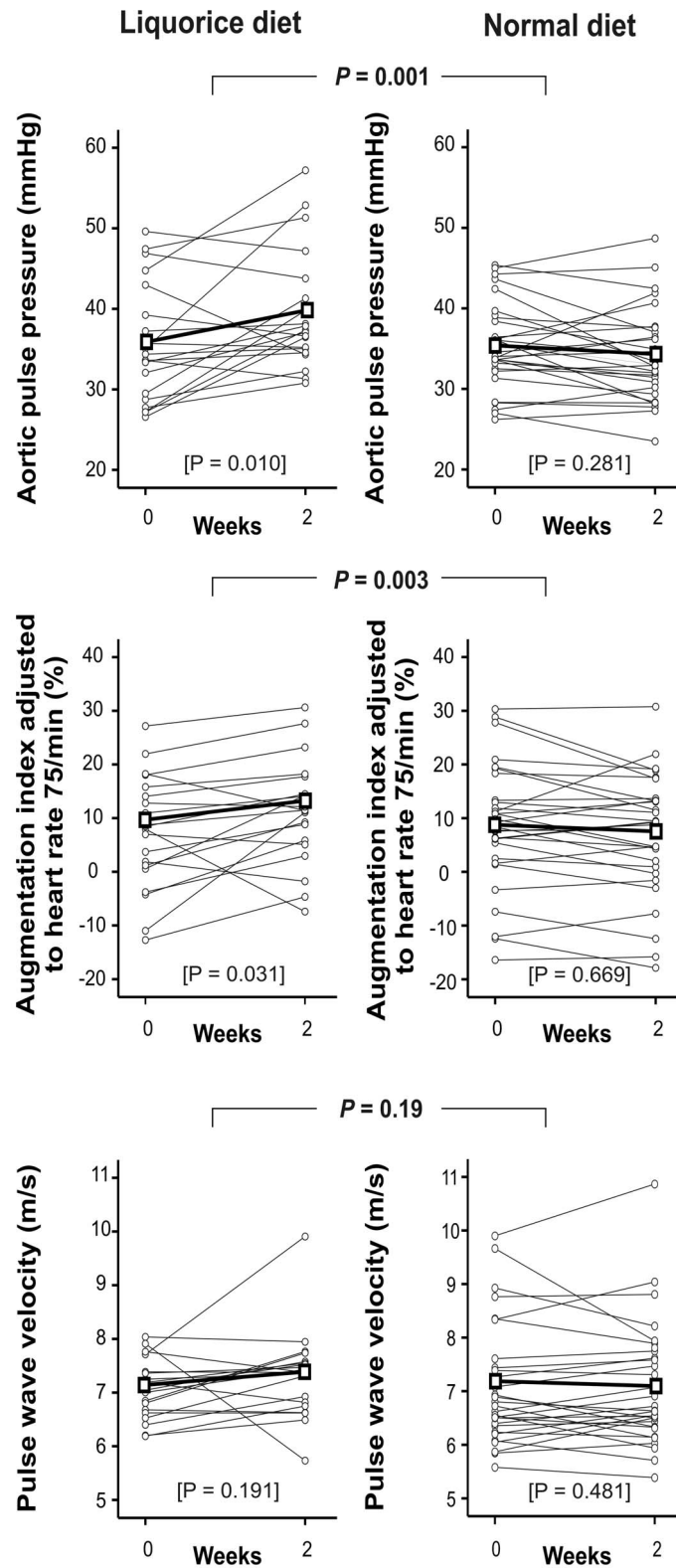


Figure 3. Aortic pulse pressure, augmentation index, and pulse wave velocity, liquorice diet versus normal diet. Grey lines represent each individual, thick black line represents mean values of each variable. P values are for the difference in the change of each variable between liquorice diet versus normal diet, P values in brackets are for the change within each group, n = 20 and 30 in the liquorice and normal diet groups, respectively.

doi:10.1371/journal.pone.0105607.g003

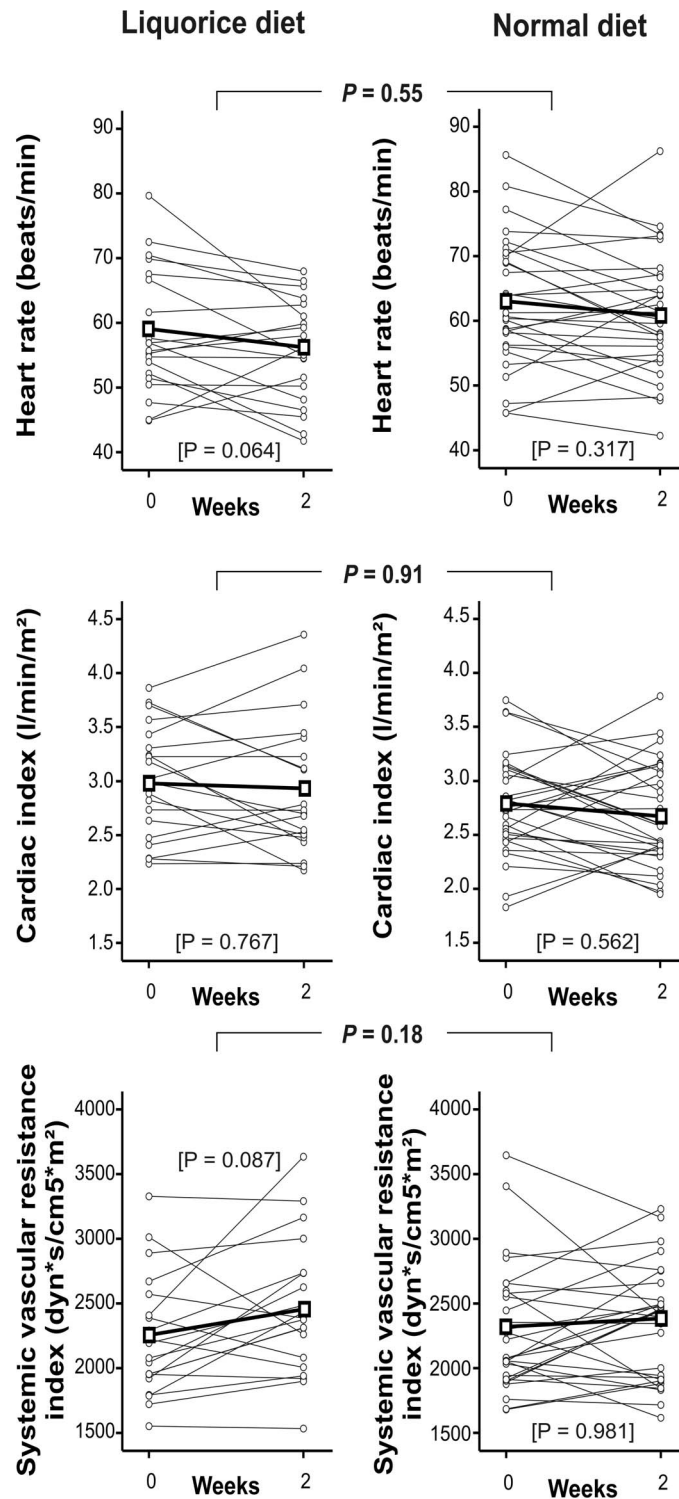


Figure 4. Heart rate, cardiac index, systemic vascular resistance index, liquorice diet versus normal diet. Grey lines represent each individual, thick black line represents mean change of each variable. *P* values are for the difference in the change of each variable between liquorice diet versus normal diet, *P* values in brackets are for the change within each group, *n* = 20 and 30 in the liquorice and normal diet groups, respectively. doi:10.1371/journal.pone.0105607.g004

dose of glycyrrhizin of approximately 21 mg per every Finnish citizen. Although glycyrrhizin containing products are exported, this indicates high domestic consumption of liquorice products.

A limitation of the present study is the rather small number of participants, although significant haemodynamic changes were observed in the 20 subjects. Due to the small number of subjects, the possible blood pressure-independent haemodynamic effects of

liquorice could not be examined by the use of multivariate analysis. However, as the effect of liquorice on blood pressure is well-established, the objective of the present study was to examine the haemodynamic changes that can explain the elevation of blood pressure. Furthermore, in addition to glycyrrhizin, the present liquorice diet also contained carbohydrates, mainly starch-derived glucose. The average intake of carbohydrates from liquorice was 150 grams per day, which could have influenced cardiovascular status. Diets with high glycaemic index are also associated with higher degree of vascular oxidative stress [39]. However, liquorice root extract contains many natural antioxidants [40], and the balance between these two factors remains unknown. The rapid 2 kg weight gain during the study was most likely due to fluid retention, since the total intake of carbohydrates from liquorice was about 2 kg during the 2-week period. The bioimpedance measurements suggested that extracellular water was increased by approximately 0.5 kg, but possible changes in the volume of intracellular water remain unknown. The observed change in weight did not correlate with changes in blood pressure, in parallel with previous findings by Sigurjonsdottir et al. showing a corresponding increase in weight following liquorice ingestion that was not associated with changes in blood pressure [12].

In order to avoid the excess carbohydrate intake, the participants could be given pure glycyrrhizin. However, we especially tested the hypothesis whether daily glycyrrhizin intake from liquorice would induce changes in cardiovascular regulation that could be detected with our recording system. As the effect of liquorice may differ between sexes, the present study subjects were chosen so that the distribution of genders in the groups did not differ. In a study concerning individual sensitivity to the harmful influences of liquorice intake, women were suggested to be more sensitive to the adverse effects of liquorice than men [12]. In contrast, a later study by the same group showed that plasma

aldosterone levels were lower in men than in women during 11 β -HSD2 inhibition with liquorice [41].

In conclusion, the results of this study with normal volunteers indicated that already two weeks of liquorice ingestion increased extracellular volume, amplified pressure wave reflection from the peripheral circulation, and elevated central systolic and diastolic blood pressure.

Supporting Information

Protocol S1 Trial protocol.
(PDF)

Protocol S2 English summary of trial protocol.
(PDF)

Checklist S1 CONSORT checklist.
(PDF)

Data S1 Leskinen data.
(TXT)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MHL IHP. Performed the experiments: MHL AMT JKK MP IHP. Analyzed the data: MHL EJH AMT AJT IHP. Contributed reagents/materials/analysis tools: AMT JKK MK TK ON JM IHP. Wrote the paper: MHL EJH AMT JM IHP. Ethical approval and registration at ClinicalTrials.gov: MHJ AMT IHP.

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PUBLICATION

III

Voluntary liquorice ingestion increases blood pressure via increased volume load, elevated peripheral arterial resistance, and decreased aortic compliance

Hautaniemi EJ, Tahvanainen AM, Koskela JK, Tikkakoski AJ, Kähönen M, Uitto M, Sipilä K, Niemelä O, Mustonen J, Pörsti IH

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Voluntary liquorice ingestion increases blood pressure via increased volume load, elevated peripheral arterial resistance, and decreased aortic compliance

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We investigated the haemodynamic effects of two-week liquorice exposure (glycyrrhizin dose 290–370 mg/day) in 22 healthy volunteers during orthostatic challenge. Haemodynamics were recorded during passive 10-minute head-up tilt using radial pulse wave analysis, whole-body impedance cardiography, and spectral analysis of heart rate variability. Thirty age-matched healthy subjects served as controls. Liquorice ingestion elevated radial systolic ($p < 0.001$) and diastolic ($p = 0.018$) blood pressure and systemic vascular resistance ($p = 0.037$). During orthostatic challenge, heart rate increased less after the liquorice versus control diet ($p = 0.003$) and low frequency power of heart rate variability decreased within the liquorice group ($p = 0.034$). Liquorice intake increased central pulse pressure ($p < 0.001$) and augmentation index ($p = 0.002$) supine and upright, but in the upright position the elevation of augmentation index was accentuated ($p = 0.007$). Liquorice diet also increased extracellular fluid volume ($p = 0.024$) and aortic to popliteal pulse wave velocity ($p = 0.027$), and aortic characteristic impedance in the upright position ($p = 0.002$). To conclude, in addition to increased extracellular fluid volume and large arterial stiffness, two weeks of liquorice ingestion elevated systemic vascular resistance and augmentation index. Measurements performed at rest may underestimate the haemodynamic effects of liquorice ingestion, as enhanced central wave reflection and reduced chronotropic response were especially observed in the upright position.

The mineralocorticoid receptor (MR)¹ and the glucocorticoid receptor (GR)² play an important role in the regulation of blood pressure (BP). Both MR and GR are expressed in several tissues important to BP homeostasis, including the kidney, vascular wall, central nervous system, and the heart^{2–4}. Cortisol binds to both GR and MR, although aldosterone is the only physiologic agonist of the MR³. In aldosterone target tissues the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) prevents cortisol from binding to the MR by inactivating it to cortisone⁵. The enzyme 11 β -HSD2 may be involved in the pathogenesis of hypertension, since decreased 11 β -HSD2 activity increases the activation of the MR and GR by cortisol⁵.

The elevation of BP after liquorice ingestion is well-known^{6–8}. The active metabolite in liquorice, glycyrrhetic acid (GA) that resembles the structure of cortisone, inhibits the enzyme 11 β -HSD2⁶. In the kidney, 11 β -HSD2 is expressed in the distal nephron, where MR activation by cortisol promotes sodium reabsorption and potassium excretion into the urine⁵. GA may also inhibit the hepatic steroid-metabolizing enzymes 5 β -reductase and 3 β -hydroxysteroid dehydrogenase, resulting in suppressed aldosterone catabolism⁹. These mechanisms lead to increased BP, sodium and water retention, decreased plasma potassium and aldosterone concentration, and decreased plasma renin activity⁶.

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The vascular wall (smooth muscle and endothelial cells)¹⁰, the heart⁴ and the brain¹¹ are also expression sites of the enzyme 11 β -HSD2. In the vascular wall, 11 β -HSD2 inhibition increases arterial tone through enhanced contractile responses to pressor hormones and decreased production of endothelial nitric oxide^{12,13}. In smooth muscle cells MR activation may contribute to vascular stiffening via remodelling of the vascular wall¹. In the heart, glucocorticoids have been suggested to induce direct effects through MR activation¹⁴. Further, the infusion of GA into the lateral ventricle of the rat brain elevates BP without affecting salt or water homeostasis, suggesting a direct influence on the central nervous system¹⁵.

We recently reported that daily liquorice consumption in normotensive subjects for two weeks elevated peripheral and central systolic and diastolic BP, increased extracellular fluid volume, and amplified pressure wave reflection from the peripheral arterial tree⁸. However, increased peripheral arterial resistance and large arterial stiffness, and changes in cardiac function and autonomic tone could also contribute to the elevation of BP following liquorice ingestion. Since the upright cardiovascular influences of liquorice exposure are also unknown, in the present study we investigated the haemodynamic changes in healthy subjects during orthostatic challenge following voluntary liquorice intake. The balance of cardiac autonomic tone was evaluated by the use of power spectral analysis of heart rate variability (HRV)¹⁶.

Methods

Ethical statement. The investigation was performed with the understanding and written informed consent of each individual, and was approved by the Ethics Committee of the Tampere University Hospital (study code R07053M) conforming to the principles outlined in the Declaration of Helsinki. The study is registered in the database of clinical trials (ClinicalTrials.gov, ID: NCT01742702, date of registration 29 of November 2012), and is a part of an investigation on noninvasive recording of hemodynamics (DYNAMIC-study; EudraCT-number 2006-002065-39).

Study subjects. The study population comprised of 52 healthy, normotensive (average brachial office BP 117/71 mmHg) individuals aged between 21 and 58 years. Exclusion criteria were office BP >140/90 mmHg, cardiovascular disease with regular medication, pregnancy, and consumption of liquorice >300 grams per week. Subject recruitment and data collection have been described previously⁸. The liquorice group consisted of 22 subjects (14 women and 8 men), and the aged-matched control group of 30 subjects (17 women and 13 men). When compared with our previous report focused on haemodynamics at rest⁸, the present analyses included data from two additional subjects in the liquorice group not included in the previous study.

As previously reported⁸, the regular medications in the liquorice group were 160 μ g budesonide + 4.5 μ g formoterol twice daily for asthma ($n = 1$), 5 mg escitalopram once daily for depression ($n = 1$), postmenopausal oestrogen replacement therapy ($n = 1$) and oral contraceptives ($n = 5$), while in the control diet group 5 female subjects used oral contraceptives and 3 had hormonal intrauterine devices. Four subjects were current smokers and 1 was previous smoker in the liquorice group, and 3 subjects were current and 4 previous smokers in the control group.

Design. The study design has been described in detail elsewhere⁸. Briefly, this was an open-label study but subjects in the control group were not aware of acting as controls for the liquorice group. In the liquorice group, the subjects consumed commercial liquorice products (Halva liquoriceTM or Kouvola liquoriceTM) daily for two weeks. Prior to baseline measurements, liquorice-containing products were not allowed for 3 weeks. During the intervention the ingested dose of liquorice was 120–300 g/d depending on the glycyrrhizin concentration of the product and the estimated dose of glycyrrhizin ranged 290–370 mg/d. All subjects maintained the liquorice diet for two weeks. In the control group, the subjects were asked to maintain their habitual diet, and the reported frequency of liquorice consumption in each subject was once per month or lower.

Physical and laboratory examinations were performed to all participants to ensure the suitability for the study, and structured questionnaires were utilized to review the lifestyle habits and medical and family history. At baseline blood and urine samples were drawn after about 12 hours of fasting with the exception that samples were not received from one subject in the control group⁸. Hemodynamic measurements were performed before and after 2 weeks of liquorice ingestion or 1–3 weeks of control diet. As exceptions, in the control diet group three subjects had consecutive measurements within 2–4 days and two subjects within approximately 4 weeks.

Laboratory analyses. Standard 12-lead electrocardiograms were recorded with MAC5000 (GE Healthcare, Chalfont St. Giles, UK), and the recordings were normal in all subjects⁸. Plasma sodium, potassium, creatinine, glucose, cholesterol lipoproteins were determined by Cobas Integra 700/800 (F. Hoffmann-LaRoche Ltd, Basel, Switzerland), and blood cell counts by ADVIA 120 or 2120 (Bayer Health Care, Tarrytown, NY, USA).

Haemodynamic measurement protocol. Preceding the haemodynamic recordings, caffeine containing products, smoking and heavy meal for at least 4 hours, and alcohol for at least 24 hours were to be avoided. The measurement protocol was conducted in a quiet, temperature-controlled laboratory by research nurses¹⁷. The impedance cardiography electrodes were placed on body surface, the tonometric sensor for radial BP on the left wrist, and a brachial cuff for BP calibration to the right upper arm¹⁸. The extended left arm was placed on an arm support at the level of the heart in supine and upright positions. The haemodynamics were recorded continuously for 5 minutes in supine position and for 5 minutes during orthostatic challenge to 60 degrees¹⁷. For the statistical analyses, mean values of each minute of the 10-min recordings were calculated.

Pulse wave analysis. Radial BP and pulse wave form were continuously captured by a tonometric sensor (Colin BP-508T, Colin Medical Instruments Corp., USA). The radial BP recordings were calibrated twice during both 5-minute periods using contralateral brachial BP measurements. Aortic BP, pulse pressure (PP) and

augmentation index (AIx) (augmentation pressure/PP*100) were determined using the SphygmoCor PWMx pulse wave monitoring system (Atcor Medical, Australia)¹⁹.

Whole-body impedance cardiography. A whole-body impedance cardiography device (CircMon^R, JR Medical Ltd., Tallinn, Estonia), which records the changes in body electrical impedance during cardiac cycles, was used to determine beat-to-beat heart rate (HR), stroke volume (SV), cardiac index (cardiac output/body surface area), pulse wave velocity (PWV), and extracellular water (ECW) volume^{20,21}. The method description and electrode configuration have been previously reported^{20,21}. Systemic vascular resistance index (systemic vascular resistance/body surface area) (SVRI) was calculated from the radial BP signal and cardiac index measured by CircMon^R. Aortic characteristic impedance was calculated as follows: (central forward wave amplitude*ventricular ejection duration)/(2*stroke volume)²².

With the CircMon^R whole-body impedance cardiography method, the recorded PWV values show excellent correlation with values measured using ultrasound or the tonometric SphygmoCor method^{18,20}, the SV shows good correlation with 3-dimensional echocardiography recordings²³, cardiac output values are in good agreement with values measured by the thermodilution method²¹, and the reproducibility and repeatability of the measurements are good^{17,23}.

Frequency domain analysis of heart rate variability. The electrocardiograms recorded by the CircMon^R device (sampling rate 200 Hz), were analyzed using Matlab software (MathWorks Inc., Natick, Massachusetts, USA). Normal R-R intervals were recognized, and a beat was considered ectopic if the interval differed over 20% from the previous values. The artifacts were processed using the cubic spline interpolation method. The frequency domain variables were calculated using the Fast Fourier Transformation method: i) power in low frequency (LF) range (0.04–0.15 Hz), ii) power in high frequency (HF) range (0.15–0.40 Hz), and iii) LF/HF ratio²⁴.

Statistical analyses. A minimum sample size of 17 experimental and 26 control subjects was required to detect a 9 mmHg difference in the change in systolic BP from baseline with a standard deviation (SD) of 10, α -level of 0.05, and power of 80%⁸. Statistical analyses were conducted using IBM SPSS Statistics Version 24 (IBM Corporation, Armonk, NY, USA). Normally distributed data was given as means with SD, standard error of the mean or 95% confidence interval. LF and HF power were transformed to natural logarithm before analyses to yield normal non-skewed distributions. The homogeneity of variances was tested with the Levene's test. The mean haemodynamic values were calculated from the minutes 3–5 of the recordings during supine and upright positions when the signal was most stable, and the values from the minutes 1–5 min were used for analyses of LF and HF power. Independent samples t-test was used to compare baseline data and changes between the groups. Analysis of variance for repeated measurements was applied to study interaction between time and group, and differences between the groups and over time in haemodynamic variables and HRV during rest and orthostatic challenge. In case of multiple comparisons, the results were adjusted with the Bonferroni correction, as appropriate. Chi-square test was applied to test non-continuous variables, and Spearman's correlations (r_s) were calculated, as appropriate. $P < 0.05$ was considered statistically significant.

Data availability. The datasets generated during and analysed during the current study are not publicly available as our clinical database contains several indirect identifiers and the informed consent obtained does not allow publication of individual patient data. However, the datasets are available from the corresponding author on reasonable request.

Results

The baseline characteristics of the study subjects are presented in Table 1. The demographic data, routine laboratory values, and ECW volumes did not differ between the groups.

The P -values in the figures refer to the changes in haemodynamic variables induced by the liquorice diet versus controls from week 0 to week 2. The other statistical results are given below in the text.

Haemodynamic variables at baseline. At week 0, there were no differences in radial systolic and diastolic BP (Fig. 1), HR (Fig. 2), aortic PP, AIx and aortic characteristic impedance (Fig. 3), or SVRI (Fig. 4) during the 10-min recordings between the groups. However, analysis of variance for repeated measurements showed a significant time*group interaction ($p < 0.001$) in the baseline analyses of SV (Fig. 2), so that the values at rest were higher in the liquorice group than in the control group. In addition, a significant time*group interaction in cardiac index ($p = 0.003$) at week 0 was observed (Fig. 4) indicating a higher upright decrease of cardiac output in the liquorice group.

Haemodynamic influences of 2-week liquorice ingestion. After two weeks of liquorice ingestion, radial systolic BP was elevated throughout the 10-min recording protocol, while an increase in diastolic BP was detected in the supine position (Fig. 1). During orthostatic challenge the increase in HR ($p = 0.003$) was reduced after liquorice consumption (Fig. 2). Increased aortic PP, AIx (Fig. 3), and SVRI (Fig. 4), were also observed after the liquorice diet. The elevation of AIx in the upright position was even accentuated after the liquorice diet, as indicated by the significant time*group interaction (Fig. 3, $p = 0.007$), and in parallel aortic characteristic impedance was increased (Fig. 3). There were no significant differences in the analyses of changes in SV (Fig. 2) or cardiac index (Fig. 4) from week 0 to 2 between the groups. Of note, the more pronounced upright decrease in cardiac index in the liquorice group persisted throughout the study (Fig. 4, $p < 0.001$ for the time*group interaction during the 10-min recording protocol at week 2).

| | Control (n = 29–30*) | | Liquorice (n = 22) | | P value |
|--------------------------------------|-------------------------|------|-----------------------|------|------------|
| | Mean | SD | Mean | SD | |
| Female/Male | 17/13 | | 14/8 | | 0.776 |
| Age (years) | 33.8 | 8.1 | 34.9 | 9.2 | 0.656 |
| Body mass index (kg/m ²) | 23.0 | 2.7 | 23.3 | 1.9 | 0.643 |
| Waist circumference (cm) | | | | | |
| Male | 86.9 | 4.4 | 82.6 | 7.1 | 0.100 |
| Female | 78.5 | 10.3 | 76.3 | 7.4 | 0.521 |
| Extracellular water (l) | 12.8 | 1.2 | 12.3 | 1.5 | 0.228 |
| Hematocrit (%) | 0.42 | 0.05 | 0.40 | 0.03 | 0.222 |
| Fasting plasma | | | | | |
| Cholesterol (mmol/l) | 4.4 | 0.8 | 4.6 | 0.8 | 0.366 |
| Triglycerides (mmol/l) | 0.94 | 0.49 | 0.83 | 0.32 | 0.374 |
| High density lipoprotein (mmol/l) | 1.72 | 0.39 | 1.83 | 0.36 | 0.305 |
| Low density lipoprotein (mmol/l) | 2.2 | 0.7 | 2.4 | 0.6 | 0.478 |
| Glucose (mmol/l) | 5.0 | 0.4 | 5.2 | 0.4 | 0.151 |
| Creatinine (μmol/l) | 76 | 11 | 77 | 17 | 0.805 |
| Sodium (mmol/l) | 140 | 2.0 | 140 | 1.3 | 0.957 |
| Potassium (mmol/l) | 3.9 | 0.2 | 3.9 | 0.3 | 0.448 |

Table 1. Demographic data and laboratory values at baseline. *Blood samples for fasting plasma values were not obtained from one subject.

The AIx is influenced by PWV, a marker of arterial stiffness, but also other factors including SVRI, HR, ventricular ejection duration, SV, and ECW volume^{8,18,25}. Therefore, we determined the correlations between these variables in the liquorice group: the increase of AIx in the upright position significantly correlated with the changes in HR ($r_s = -0.592$, $p = 0.004$), SVRI ($r_s = 0.600$, $p = 0.003$) and ejection duration ($r_s = 0.736$, $p < 0.001$), but not with the changes in PWV, SV or ECW volume. Since the number of the study subjects was rather small, the use of multivariate analysis for further statistics was not feasible.

At week 2 the mean changes in ECW volume were -0.16 litres (95% CI -0.37 to 0.04) versus $+0.57$ litres (95% CI -0.03 to 1.17) in the control versus liquorice groups, respectively ($p = 0.024$ for between group difference). The PWV mean (SD) values at baseline were 7.1 (1.1) and 7.1 (0.7) m/s in the control and liquorice groups ($p = 0.880$), respectively. Although the mean changes (95% CI) in PWV were small within the control and liquorice groups; -0.18 m/s (-0.38 to 0.03) versus 0.27 m/s (-0.11 to 0.65), respectively, these changes were significantly different between the groups ($p = 0.027$).

Heart rate variability after 2-week liquorice ingestion. HRV is dependent on average HR due to both physiological and mathematical reasons²⁶. The mathematical dependency results from the nonlinear relationship between RR interval and HR, and as a consequence of this association the HRV analysis may be biased²⁶. In order to reduce the HRV dependence on HR, we divided the LF and HF power by the average RR interval squared²⁶. In the HR-corrected power spectral analyses of HRV, there were no significant within-group changes in the HF power or LF/HF ratio either in supine or upright positions (Fig. 5, $p > 0.05$ for all comparisons). Although there were no significant between-group differences in LF power, in the liquorice group the LF power was decreased after two weeks (Fig. 5, $p = 0.034$). As a minor between-group difference, the upright LF/HF ratio was lower in the liquorice group than in the control group after two study weeks (Fig. 5, $p = 0.008$).

Discussion

The liquorice-induced elevation of BP has been attributed to renal sodium and water retention⁶. In line with these views, our previous findings indicated that the elevation of BP after liquorice exposure was associated with increased extracellular fluid volume⁸. In the present study, we investigated the haemodynamic influences of two-week liquorice intake during orthostatic challenge, and found that the exposure increased extracellular fluid volume and elevated radial systolic and diastolic BP, SVRI, aortic PP, and AIx. In addition, liquorice diet resulted in significant changes in PWV and aortic characteristic impedance, two indexes of aortic stiffness^{22,25}, when compared with the control group. During orthostatic challenge, liquorice ingestion resulted in a further increase of AIx indicating enhanced pressure wave reflection from the periphery, while a decreased cardiac chronotropic response was also observed. In the power spectral analyses of HRV, the LF power was decreased in the liquorice group after two weeks. Altogether, the present results indicate that the liquorice-induced elevation of BP was due to multiple mechanisms: volume overload, reduced large arterial compliance, increased peripheral vascular resistance, and enhanced pressure wave reflection from the periphery particularly in the upright position.

BP is defined as the product of cardiac output and peripheral vascular resistance²⁷. The present liquorice and control groups demonstrated persistent functional differences in the regulation of cardiac output in the upright position. However, the between-group difference in cardiac index remained unchanged during the study, while

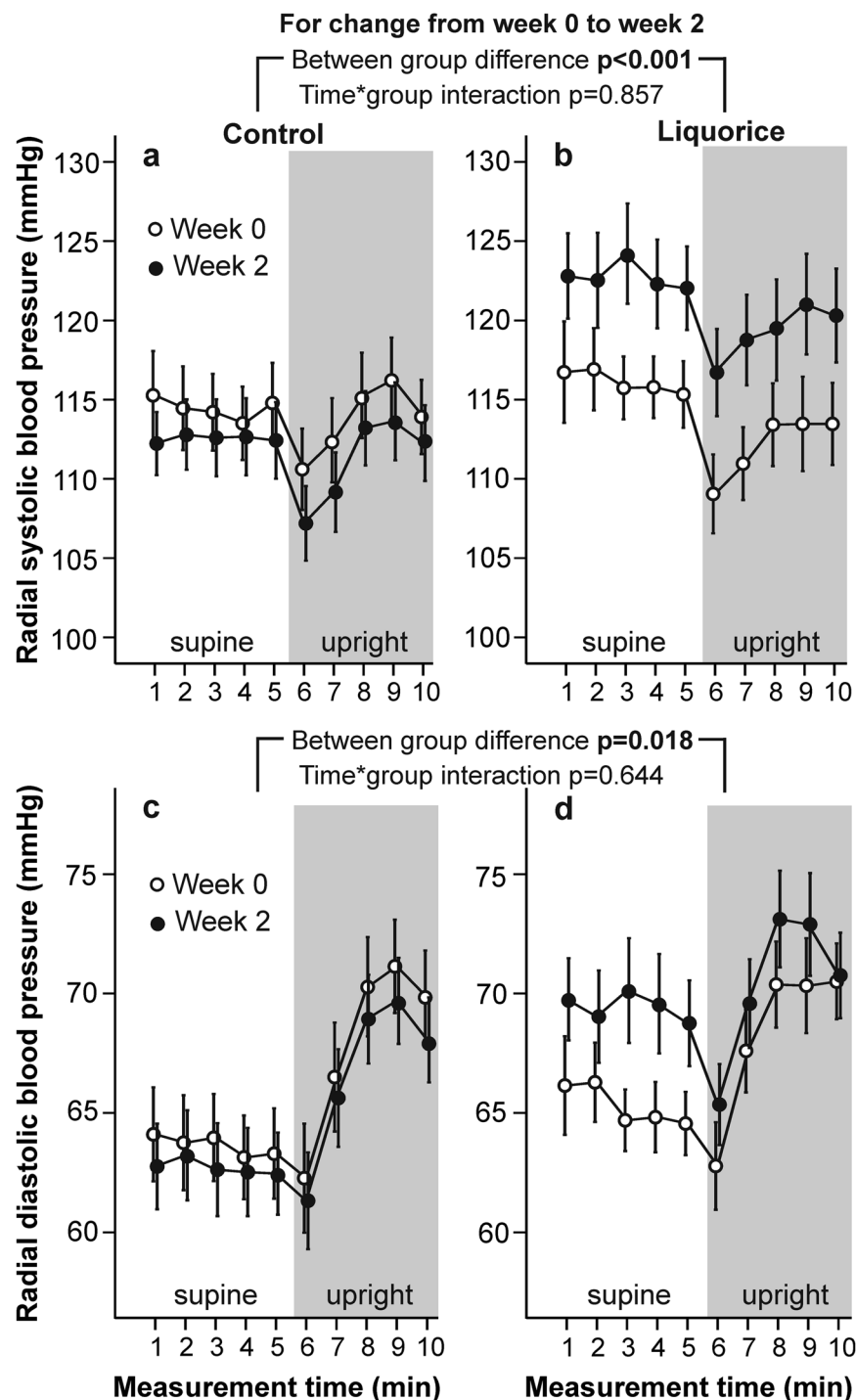


Figure 1. Radial systolic blood pressure (a,b) and diastolic blood pressure (c,d) before and after two weeks of control and liquorice diet. Haemodynamic data was captured continuously during the 10-min recordings, and passive head-up tilt was performed from 5 to 10 min. The graphs depict mean and standard error of the mean, and the statistical analyses are for the changes in the liquorice group versus controls from week 0 to week 2.

SVRI was elevated after liquorice ingestion. Several mechanisms may explain the observed increase in systemic vascular resistance after the liquorice diet. Glucocorticoid activity is controlled locally and systemically by the type 1 and 2 isoforms of enzyme 11β -HSD⁵. Both 11β -HSD enzymes are expressed in the vascular wall, where they can influence vascular tone by regulating active glucocorticoid concentrations¹³. The subsequent MR activation in smooth muscle cells can result in the remodelling of the vascular wall¹. Glucocorticoids can even potentiate the vasoconstrictor actions of angiotensin II and catecholamines in smooth muscle, and suppress vasodilatory systems including endothelial nitric oxide synthase and prostacyclin synthesis²⁸. The activation of GR may reduce

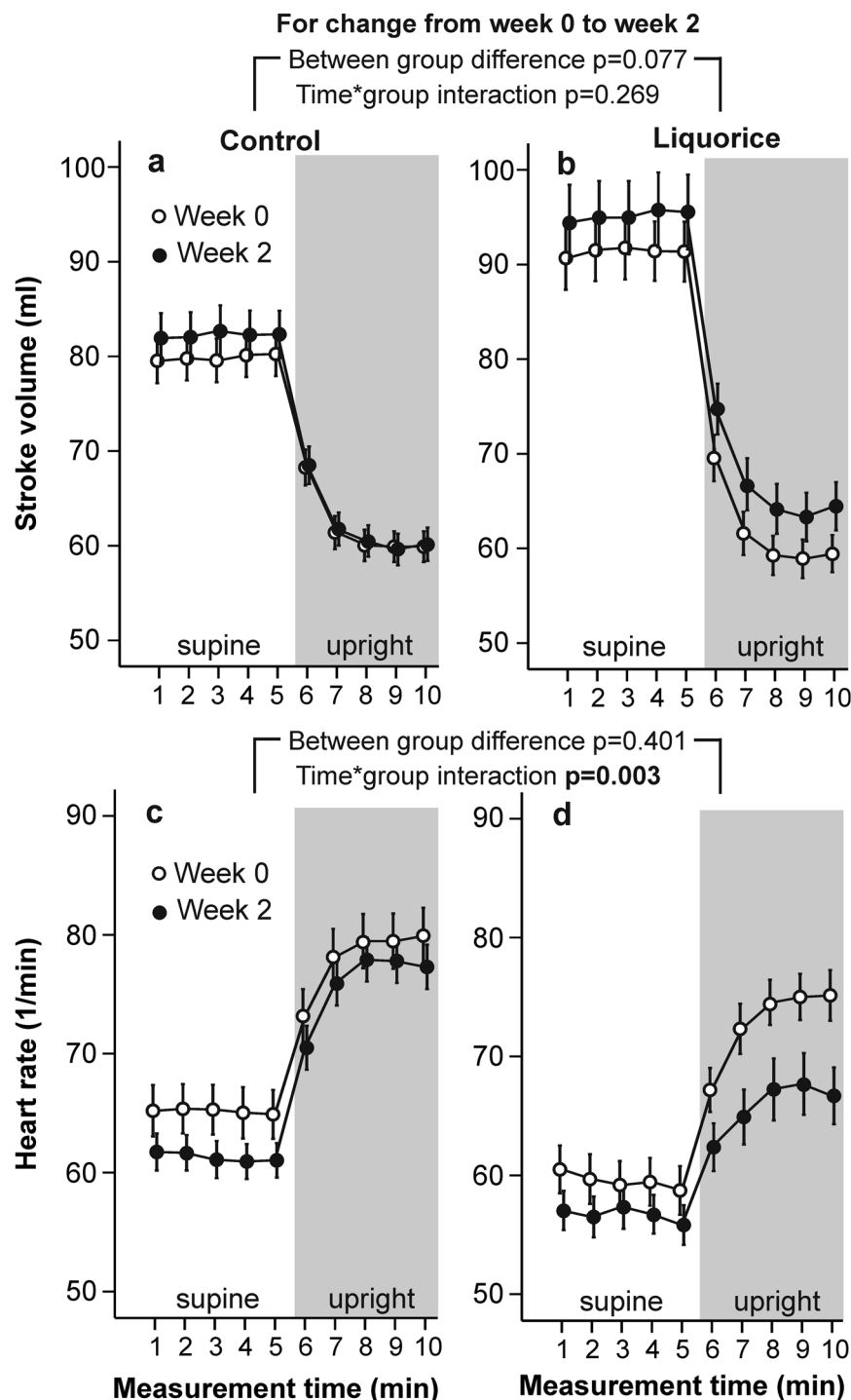


Figure 2. Stroke volume (a,b) and heart rate (c,d) before and after two weeks of control and liquorice diet. Mean and standard error of the mean, statistical analyses are for the changes in the liquorice group versus controls from week 0 to week 2.

neuronal nitric oxide release in the arteries²⁹, whereby alterations in perivascular nitrergic function may also contribute to the glucocorticoid-induced increase in vascular resistance.

Autonomic nervous system plays a significant role in the control of BP¹⁶. During postural challenge, a decrease in BP is sensed by the baroreceptors of carotid sinus and aortic arch, and afferent input to the nucleus of the tractus solitaries is decreased¹⁶. Subsequently, efferent vagal input to the sinoatrial node is reduced, sympathetic input to the heart, arterioles and venules is increased¹⁶, and the reduction in BP is corrected via increases in HR and systemic vascular resistance¹⁶. In the present study, the HR response to orthostatic challenge was attenuated after liquorice ingestion when compared with the control diet. Simultaneously, the LF power that predominantly reflects sympathetic activity²⁴, was decreased in the liquorice group. Also, the LF/HF ratio, an indicator of cardiac

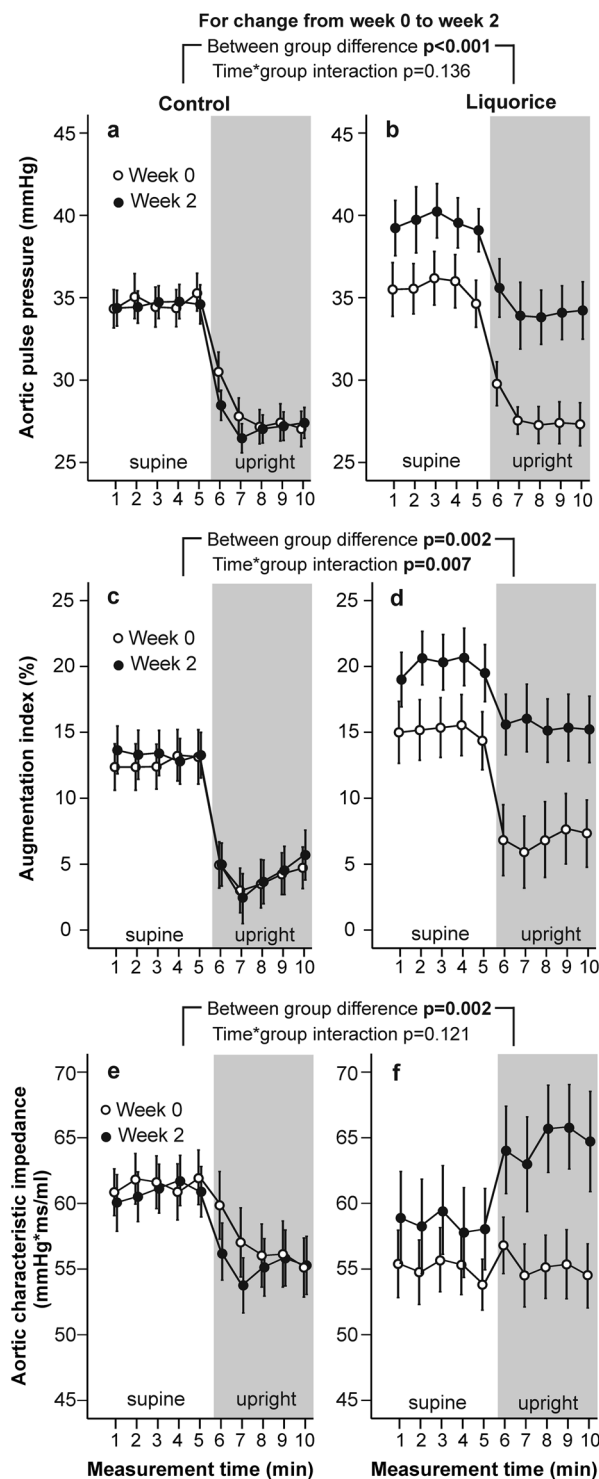


Figure 3. Aortic pulse pressure (a,b), augmentation index (c,d), and aortic characteristic impedance (e,f) before and after two weeks of control and liquorice diet. Mean and standard error of the mean, statistical analyses are for the changes in the liquorice group versus controls from week 0 to week 2.

sympathovagal balance²⁴, was slightly lower in the liquorice group than in controls at week 2. The results of the power spectral analyses were in line with the observed changes in the control of HR after liquorice ingestion, and indicate that there was no increase in cardiac sympathetic tone during the intervention. Therefore, the alterations in sympathovagal balance were not the cause for the liquorice-induced elevation of BP. Previous experimental findings suggest that the changes in cardiac function could result from direct MR or GA action in the heart, as aldosterone has been found to decrease heart rate and repolarization rate in rabbit heart muscle cells³⁰, while GA has been reported to show a negative inotropic action in the isolated perfused rat heart³¹.

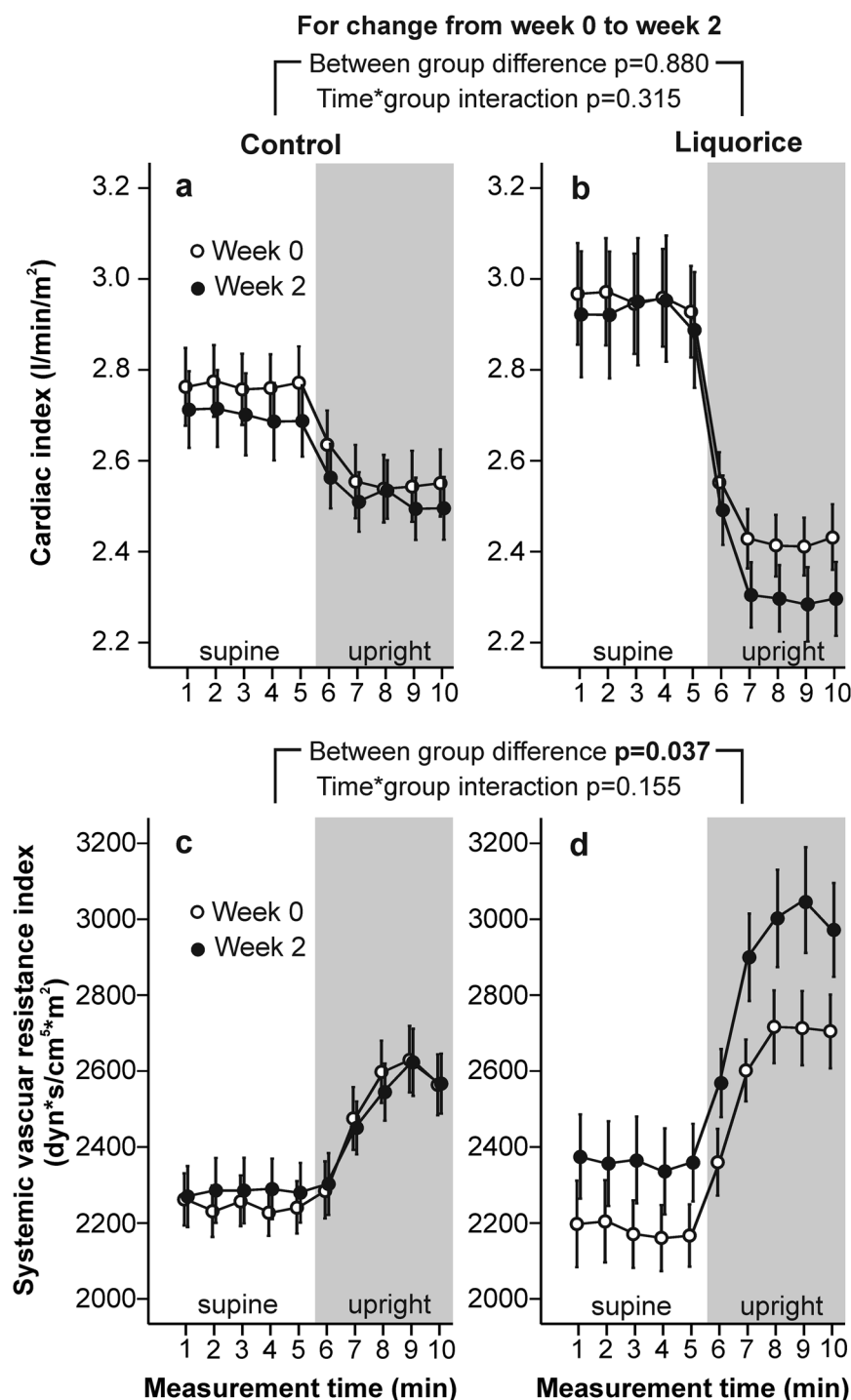


Figure 4. Cardiac index (a,b) and systolic vascular resistance index (c,d) before and after two weeks of control and liquorice diet. Mean and standard error of the mean, statistical analyses are for the changes in the liquorice group versus controls from week 0 to week 2.

The determination of PWV is considered the gold standard in the evaluation of arterial stiffness²⁵. The present within-group changes in PWV after the liquorice and control diets were not significant, but the changes in PWV between the two groups were significantly different. We also evaluated large arterial stiffness by calculating the aortic characteristic impedance, i.e. the impedance to the left ventricular pulsatile flow²². Increased aortic stiffness increases the characteristic impedance and forward wave amplitude as a result from the mismatch between properties of the aortic root and peak aortic flow²². Although forward wave amplitude mainly represents the blood flow going forward from the heart, the results must be interpreted with caution, as wave reflection from the peripheral circulation may also influence the magnitude of forward wave amplitude³². We found that liquorice ingestion increased aortic characteristic impedance especially in the upright position. The present results

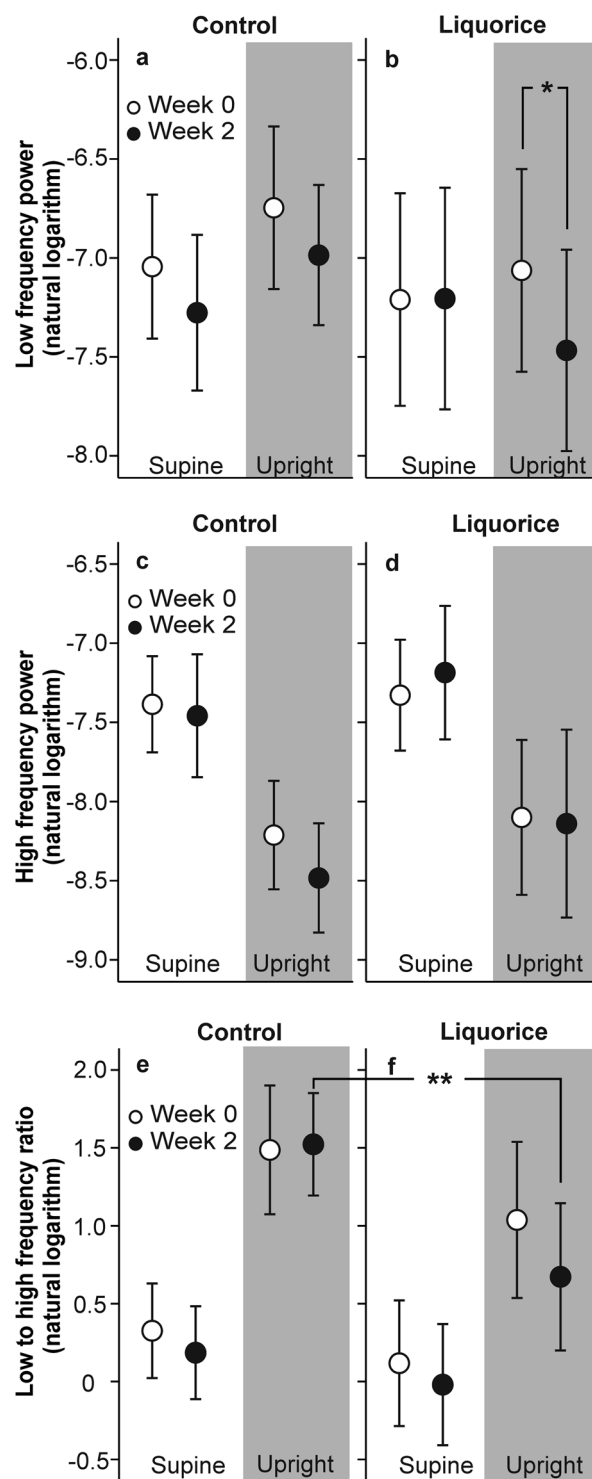


Figure 5. Low frequency power (a,b), high frequency power (c,d) and low to high frequency power ratio (e,f) before and after two weeks of control and liquorice diet in the supine and upright position. Mean and 95% confidence interval depicted, * $p = 0.034$ for the change within the liquorice group, ** $p = 0.008$ for the difference in mean values at week 2 between the groups; analysis of variance for repeated measurements, post-hoc t-tests were adjusted with Bonferroni corrections for multiple comparisons.

thus suggest that already two weeks of liquorice ingestion can increase large arterial stiffness. This is probably explained by the elevation of BP, as the indices of arterial stiffness significantly depend on the prevailing BP that is distending the blood vessels²⁵. It is unlikely that structural changes would take place during two weeks of liquorice exposure, but such changes are bound to result from longer periods of elevated BP²⁷.

The backward pressure wave that is reflected from the branches of the arterial tree and terminations of low resistance arteries into high-resistance arterioles is characterized by the AIx^{18,25,33}. The magnitude of AIx is influenced by arterial stiffness, but also by other factors including systemic vascular resistance, gender and height^{18,25}. In addition, AIx is reduced by approximately 4% for every 10 beats/min increase in HR³³. In the present study, liquorice ingestion increased the AIx especially in the upright position, i.e. the upright reduction in the level of AIx was lower after liquorice exposure. This elevation of AIx during orthostatic challenge correlated with parallel changes in HR, SVRI and ejection duration, and all of these haemodynamic alterations have the potential to increase the magnitude of AIx. Of note, after liquorice withdrawal the duration of the suppressive effect of GA on 11 β -HSD2 and the renin-angiotensin-aldosterone axis can be prolonged, since the normalization of increased urinary excretion of cortisol can take 2 weeks, while the recovery of suppressed plasma renin and aldosterone levels may last up to 2–4 months⁶.

The strength of the present investigation was in the detailed cardiovascular reactivity tests that enabled the detection of the liquorice-induced changes in supine and upright haemodynamics. The length of the present intervention can be considered to be adequate, since a maximal rise in BP has been demonstrated after 2 weeks of liquorice ingestion in study that also showed a linear dose-response relationship on the BP effect⁷. The present dose of glycyrrhizin (290–370 mg/day) did not exceed the daily dose of 400 mg, when the risk of adverse events has been found to increase in most individuals³⁴. The study population was homogenous with normal average BMI, and the distribution of genders did not differ between the study groups, which excludes the possible confounding of the previously demonstrated differences between the sexes in the effects of liquorice ingestion³⁵.

As a limitation of our study, the number of study subjects was rather small, and detailed testing of the determinants of AIx was not possible by the use of multivariate analysis. In this open-label study our aim was to test the effect of glycyrrhizin intake from liquorice on cardiovascular function, and therefore the effects of pure glycyrrhizin were not examined. A double-blinded crossover study design could not be applied, as no products with authentic liquorice taste in the absence of glycyrrhizin are available. All study subjects were instructed to maintain their habitual background diet during the study, but the stability of the dietary habits was not monitored. Of note, the participants in the control diet group were not aware of acting as controls for the liquorice study, thus among them the habitual liquorice consumption could not be unknowingly changed. Finally, the commercial liquorice product increased the intake of carbohydrates by an average of 150 grams per day corresponding the energy intake of 600 kcal per day. We have previously reported that the weight gain was 1.5 kg with simultaneous increase in ECW volume of 0.5 kg after two weeks of liquorice ingestion⁸. However, the increase in body weight did not explain the elevation of BP⁸. We have also shown that the whole-body bioimpedance PWV values correlate very well with values measured using ultrasound or the tonometric method^{18,20}.

In conclusion, this study demonstrated that in addition to extracellular fluid volume expansion, two-week liquorice exposure increased large arterial stiffness, systemic vascular resistance, and enhanced central wave reflection from the peripheral circulation. Measurements performed only at rest may result in underestimation of the haemodynamic effects of liquorice ingestion, since reduced chronotropic response and enhanced central wave reflection were especially observed in the upright position.

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Author Contributions

I.H.P. conceived and designed the study; A.M.T., J.K.K. and I.H.P. performed the experiments; E.J.H. analysed the data and interpreted the results with A.J.T. and I.H.P., E.J.H., A.M.T., J.K.K., A.J.T., M.K., K.S., O.N., J.M. and I.H.P. contributed to the collection of data, setup of the haemodynamic recording equipment, and laboratory analyses; M.U. crafted the computer programme for the HRV analyses; E.J.H. and I.H.P. drafted the first version of the manuscript. All authors provided intellectual input and contributed to the final version of the manuscript.

Additional Information

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

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PUBLICATION IV

**Liquorice ingestion attenuates nitroglycerin-induced vasodilatation and
impairs cardiac chronotropic response to upright posture**

Hautaniemi EJ, Tikkakoski AJ, Eräranta A, Kähönen M, Hämäläinen E, Turpeinen
U, Mustonen J, Pörsti IH

Submitted

