Are osteoporosis and hypertension part of the same aging process?

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Table of Content

English abstract

Introduction: Hypertension and osteoporosis are two age related diseases contributing an important health burdens on our aging society. We set to unveil a potential link between these two pathologies in a human cross-sectional study (CARTaGENE) and in an animal model study (the recombinant inbred strain of rats). ***Design and methods:*** CARTaGENE is constituted of 20,007 subjects, aged between 40 and 70 years. Subjects went through a three hour detailed phenotyping session that included peripheral and central blood pressure, height and bone mineral density (BMD), that were analysed in our project. Two additional cohorts were used for confirmation of the height and hypertension relationships, the Canadian Heart Health Surveys (N=23,129) and a family cohort of the Saguenay Lac St-Jean population (N=849 from 120 families). In the rat project, we carried our experiments on the recombinant inbred strains of rats (RIS); available strains were used in addition to one parental strain, the sponateously hypertensive rat (SHR). Radiotelemetry, bone scanning, oral glucose tolerance tests in addition to blood and urine biochemistry analysis were carried at 3, 6, 9 and 12 months of age in male and female rats. ***Results:*** Human data showed that hypertension is significantly associated with shorter stature in elderly; in addition we observed a significant association of arterial stiffness with an increased rate of fractures and low BMD. In the rat model we observed a differential display of hypertension, bone density and insulin resistance among strains and between sexes. A significant association between bone volume traits and chromosome X was found. Moreover, a significant interaction between chromosome 1 and X was found to be associated with trabecular

number. ***Conclusion:*** Data of both our studies suggest a strong link between hypertension and low bone mineral density suggesting shared genetic determinants.

French abstract

Introduction: L'hypertension et l'ostéoporose, deux maladies liées à l'âge présentent de lourds fardeaux sur le système de santé due au vieillissement de la population. Dans cette étude on a évalué le lien possible entre ces deux pathologies dans une étude populationnelle (CARTaGENE) et une étude de modèle animal (les souches des rats recombinants consanguins (RIS)). ***Matériels et méthodes:*** La cohorte CARTaGENE est composée de 20,007 sujets, âgés de 40 et 70 ans. La mesure de la pression artérielle centrale et périphérique, la mesure de la taille et de la densité osseuse étaient parmi les phénotypes recueillis auprès des participants et sont le sujet de notre analyse. Deux cohortes supplémentaires ont été utilisés afin de confirmer l'hypothèse concernant la relation entre la taille et l'hypertension, les deux cohortes étant : "Santé du cœur, Canada" (N = 23,129) et la cohorte familiale de la population du Saguenay-Lac St- Jean (N = 849 membres de 120 familles). Dans le projet des rats, nous avons entamé les expériences sur les RIS sur 3 souches disponibles en plus de la souche parentale, les rats spontanément hypertendus (SHR). La télémétrie, l'étude des paramètres osseux, le test de tolérance au glucose ainsi que l'analyse biochimique du sang et des urines ont été réalisés à 3, 6, 9 et 12 mois d'âge chez les rats mâles et femelles. ***Résultats:*** les données humaines ont montré que l'hypertension était significativement associée à une taille plus courte chez les sujets âgés; en plus nous avons observé une association significative entre la rigidité artérielle et un taux élevé de fractures ainsi qu'une plus faible densité osseuses. Dans le modèle de rat, nous avons observé une expression différentielle de l'hypertension, de la densité osseuse et de la résistance à l'insuline entre les souches et

entre les sexes. Une association significative entre les traits du volume osseux et des locus sur le chromosome X a été trouvée. De plus, une interaction significative entre le chromosome 1 et X a été trouvée associée avec le nombre de trabécules. ***Conclusion:*** Les données de nos deux études suggèrent un lien fort entre l'hypertension et la faible densité osseuse, suggérant un lien génétique partagé.

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Preface

The current thesis presents original work concerning hypertension and osteoporosis. To our knowledge it is the first study that evaluates the relation linking height, arterial stiffness, hypertension, bone mineral density and fractures in a cross-sectional population based cohort (CARTaGENE) of 20 007 subjects representing Quebec's population. In addition our animal model project represents genuine research in the field of hypertension and bone mineral density; we are the first to assess the genetic link between those two pathologies in the recombinant inbred strains of rats.

Authors contribution

CARTaGENE

Dr. Ondřej Šeda, Dr. Lucie Šedová, Dr. Pierre Dumas, Dr. Ramzan Tahir contributed to the launching of the CARTaGENE project.

Dr. Michel Joffres provided access to the Canadian Heart Health Study cohort.

Rat project

Dr. Louis-Georges Ste-Marie and Dr. Natalie Dion helped with the histomorphometric study of the rat bones.

Dr. Ondřej Šeda helped in the genetic analysis of the rat project.

Rana El Bikai did all the experiments, analysis and manuscript writing of the rat project. She also analysed and helped in the quality control of CARTaGENE's data, and wrote the manuscript.

Dr. Pavel Hamet is the principal investigator and supervised both projects.

Abbreviation list

List of figures

Introduction

Cardiovascular diseases with hypertension as its leading risk factor, accounted for 9.4 million of death worldwide in 2010 (1). Deaths due to cardiovascular diseases are projected to increase with the years if appropriate measures are not considered (Word Health day 2013) (2). Osteoporosis is a major public health concern, that affects 200 million people worldwide and is the cause of more than 8.9 million fractures annually (3, 4). It has long been known that high blood pressure, often occurs with abnormal calcium handling; a higher urine calcium excretion for a given sodium intake, an increase in the secondary parathyroid activity, an increase in intestinal calcium absorption, a low ionised serum calcium etc (5). The long term interest in our group was in the calmodulin activity in hypertension. Calmodulin is known to play an important role in actin-myosin interactions and thus being a key player in the regulation of smooth muscle tone (6). Calmodulin is observed to have an increased activity in spontaneously hypertensive rats (SHR) especially in the heart and kidney as compared to their normotensive controls. We previously showed that calmodulin activator was responsible for its enhanced activity in hypertensive rats (7). In a second study of ours, we observed that rats receiving a high calcium diet had a blood pressure reduction and a parallel decrease of calmodulin activator to almost the same levels of their normotensive controls (8). In a study that aimed at understanding the disturbance in calcium metabolism, it was demonstrated that young Dahl sensitive rats had a reduced blood pressure when fed on high calcium diet. Whereas the same diet increased the development of hypertension in the same rats at an older age, suggesting that the beneficial role of calcium supplement

is dependent on age (9). Some studies done on SHRs show that hypercalciuria and hyperparathyroidism lead to a decrease in bone mineral content (10, 11). In a study done by Metz et al. it was observed that SHR rats developed a lower cortical bone density, a lower bone calcium and magnesium content compared to their healthy controls the Wistar-Kyoto rats (12). The analysis of the prospective study of Osteoporotic Fractures done by Cappuccio et al. showed that increased blood pressure in elderly white women is associated with increased bone loss at the femoral neck (13). Andreassen et al. recently identified common genetic variants in blood pressure that are associated with other phenotypes. Among the significant single nucleotide polymorphism that were associated with systolic blood pressure were the ones associated with bone mineral density and height (14). In some of the unpublished data of the Canadian Heart Health Study (CHHS) that was carried from 1986 to 1992 on 23,129 subjects, we observed that hypertensives had a higher body height at a younger age (18-49) compared to normotensives and a shorter stature at an older age (50-74). The slope of height decline was significantly steeper in hypertensives in comparison to normotensives. The same pattern of more pronounced height decline in hypertensives was seen in the family cohort of the Saguenay Lac Saint Jean (SLSJ) (http://www.pulsus.com/ccc2010/abs/065.htm) (Manuscript 1). Those families had at least one sib pair affected with early-onset of hypertension and/or dyslipidemia. The current project entails two sub-studies: a human population project and an animal study. The first project is carried on a cross-sectional cohort (CARTaGENE) that is composed of 20,007 subjects. In this study, we validated our observation from the CHHS

and the SLSJ and assessed whether the same pattern of shorter stature in elderly hypertensive is seen. In a second step we investigated if there is a relation between body height and T-score (a measure of bone mineral density). We then tested for a possible relation between hypertension, low BMD and fractures. And finally, we evaluated if peripheral or central cardiovascular markers are best associated with low BMD and fractures.

The second study was performed in a genetically well-defined rat model, the recombinant inbred strains of rats, which display a polygenic structure of essential hypertension. The aim of the latter is to test whether those rats develop a low bone mineral density (low BMD) and insulin resistance in subsequently to hypertension while ageing.

A. Hypertension

Hypertension is the leading cause of cardiovascular diseases and mortalities in Canada and worldwide. Elevated blood pressure is most commonly defined as a systolic blood pressure of 140 mmHg or greater, and/or a diastolic blood pressure of 90 mmHg or greater, and/or are taking medications for blood pressure (15). The prevalence of hypertension increases with age and is different among races and socioeconomic groups. For instance in the United States, the rate of elevated blood pressure is higher in blacks than in whites, and is higher in less educated people than the more educated ones and that is true for both races (16). The occurrence of hypertension also differs between sexes and is more prevalent in men compared to women, particularly in Caucasians. The Canadian Heart Health Survey, reported an overall prevalence of

hypertension of 22% in the Canadian population, it was higher among men (26%) than in women (18%). The rate increased with age and hypertension became more prevalent in women compared to men in the age group of 65 to 74 (17).

1. The monogenic or Mendelian form of hypertension

Different are the causes of hypertension, some are genetic; other are environmental and in most instances it is the combination of genes and environment factors. Monogenic hypertension is rare but has an identifiable cause on the contrary of essential hypertension where the causes are usually hard to determine and are in most cases the result of genetic and environmental interactions. Three main forms of Mendelian hypertension exist: the Glucocorticoid-Remediable Aldosteronism, Liddle's syndrome and Apparent Mineralocorticoid Excess (Figure 1).

a. Glucocorticoid-Remediable Aldosteronism (GRA)

GRA is an autosomal dominant form of hypertension that affects the 11β-hydroxylase promoter regulatory region and the gene responsible for aldosterone structure (18). Lifton et al. identified a chimeric gene in which the 5-prime regulatory sequences of the CYP11B1 gene were fused to the coding region of the CYP11B2 gene (610613.0002), resulting in ectopic expression of aldosterone synthase in the zona fasciculate of the adrenal cortex (19). This gene fusion causes an elevated level of aldosterone thus leading to salt retention and volume expansion and a low plasma rennin.

b. Liddle's syndrome

It's an autosomal dominant mutation affecting the amiloride-sensitive epithelial sodium channel hence increasing the sodium channel activity. Liddle's syndrome is

characterised by hypokalemia and a suppression of plasma rennin and aldosterone activity (20).

c. Apparent Mineralocorticoid Excess

It's an autosomal recessive mutation in the gene responsible for the synthesis of 11β-hydroxysteroid dehydrogenase. Under normal conditions this enzyme converts cortisol to its inactive metabolite cortisone. This mutations causes cortisol to occupy the mineralocorticoid receptor at the distal nephron and leading to sodium and water retention in addition to low rennin and aldosterone and volume expansion (21-23).

2. Essential hypertension: a multifactorial and polygenic disease

a. The role of kidneys in hypertension

Kidneys are key players in the development of hypertension; important mechanisms underlying hypertension are mediated through the kidneys such as abnormal sodium excretion, renal vasoconstriction, renin secretion, Arginin-Vasopressin effects etc... Different suggestions were made to explain the reduced sodium excretion in cases of hypertension such as abnormalities in the Na^+-K^+-ATPase, Na^+-H^+-exchanger, Na^+-K^+-cotransporter (24). Other explanations emerged to clarify the causes of renal vasoconstriction leading to essential hypertension. The endothelium is one of the major player behind vasoconstrictions and vasodilations, it is responsible for the production of endothelin, thromboxane (vasoconstrictive agents) in addition to prostacyclin and nitric oxide (vasodilator agents) any imbalance between these molecules can cause vasoconstriction (25).

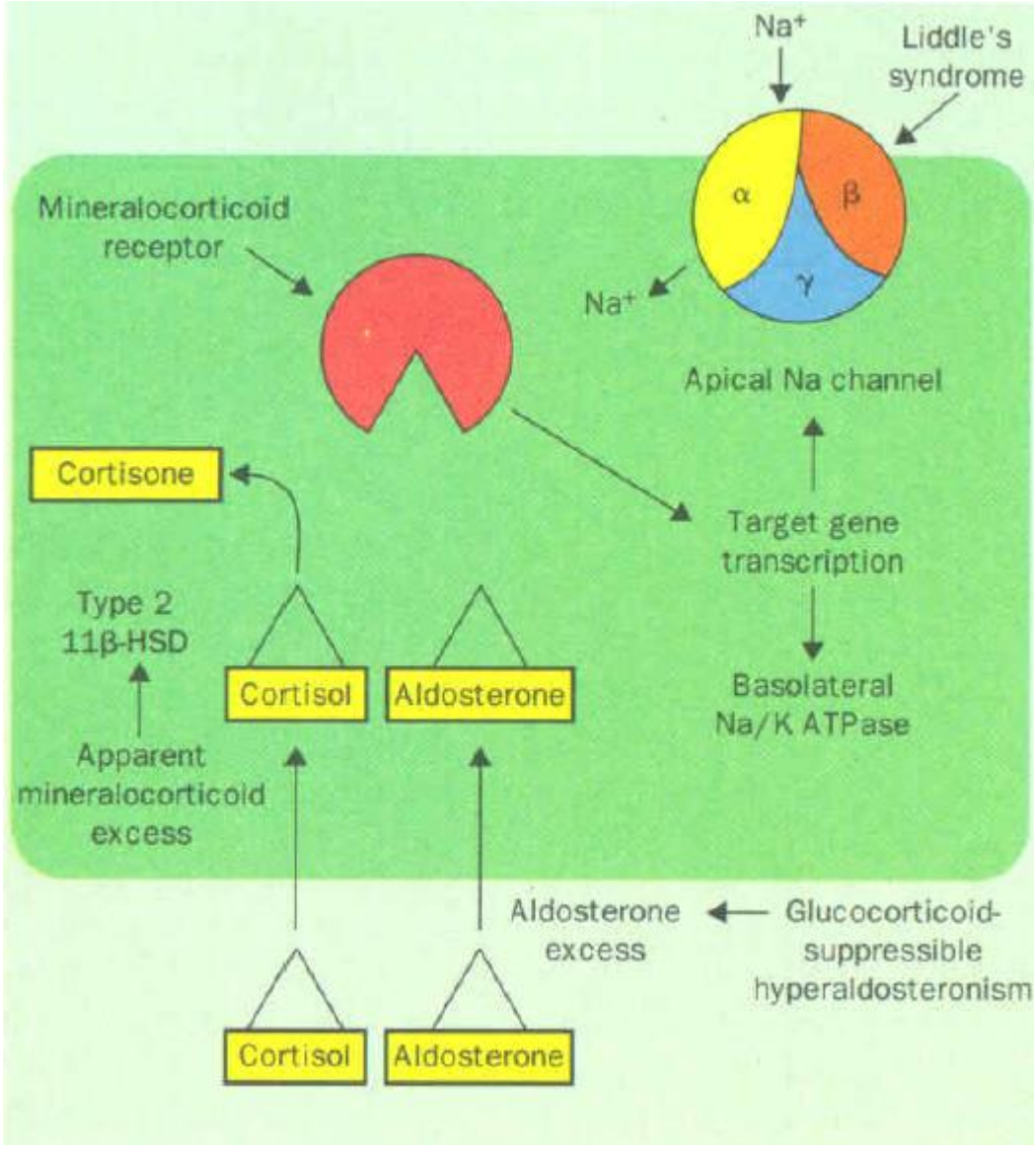

Figure 1 : Schematic representing the different monogenic forms of hypertension (22).

Different animal studies have shown the implication of the endothelium products in hypertension. For instance, salt-sensitive Dahl rats had a reduced blood pressure after administration of L-arginine showing the insufficient nitric oxide production (26). In another study, spontaneously hypertensive rats were seen to have an increase in the level of vasoconstrictor agents such as eicosanoid, despite their normal level of nitric oxide showing the imbalance between the molecules thus participating in the development of hypertension (27). Another factor that impacts the endothelium dependent pathway such as vasodilation is the balance between sodium and potassium. For example, sodium retention inhibits the synthesis of nitric oxide and increase the synthesis of its endogenous inhibitor thus impacting the vasculature (28). A factor that plays a major role in sodium reabsorption and renal vasoconstriction is the sympathetic nervous system (SNS) (Figure 2). It has been shown that hypertensive patients have a high sympathetic activity compared to normotensives. This high neural activity underlies different phenomenon observed in hypertension for example an increase in renal blood flow thus perturbing the proper sodium excretion (29) and it also affects peripheral vascular resistance and could lead to smooth muscle hypertrophy (30). Additional feature that is often seen in hypertensive patients is the impaired ability to suppress angiotensin II expression thus leading to a continuous renal vasoconstriction. In case of the latter, the rennin-angiostensin-aldosterone system is activated and an increased sodium reabsorbtion occurs (31) leading to the development of hypertension.

b. Renin-Angiotensin-Aldosterone System (RAAS)

RAAS is a hormonal system that controls blood pressure, intravascular volume, inflammatory and proliferative mechanisms. A low blood flow, a decreased sodium delivery to the kidneys or an activation of the SNS, activates the secretion of renin by the juxtaglomerular cells (32, 33). Angiotentinogen produced by the liver is then cleaved by plasma renin and generates angiotensin I (Ang I). The latter is then converted by Angiotensin Converting Enzyme (ACE) in the lungs to produce angiotensin II (Ang II) which mediates its vasoconstrive, proliferative and inflammatory effects through Angiotensine Receptor I (AT1). Activation of AT1 promotes endothelial dysfunction, smooth muscle cell proliferation, atherosclerosis and vascular hypertrophy (34). Ang II acts also on the adrenal cortex and the posterior pituitary thus stimulating the secretion of aldosterone and arginine-vasopressin respectively; in normal conditions RAAS plays a beneficial and protective role for the body to maintain a normal blood pressure. But its chronic activation leads to detrimental outcomes for the cardiovascular system. In hypertension, RAAS is activated continuously thus resulting in high concentrations of renin and Ang II. The latter exerting their detrimental effect on the vasculature causing vasoconstriction and peripheral vascular resistance. Ang II causes also volume expansion through sodium retention (via aldosterone and renal vasoconstriction). Finally, the chronic activation of RAAS leads to a build-up of negative effects eventually causing hypertension. On the other hand, low renin levels also perturb the stability of blood pressure and it is therefore called Low Renin Hypertension (LRH).

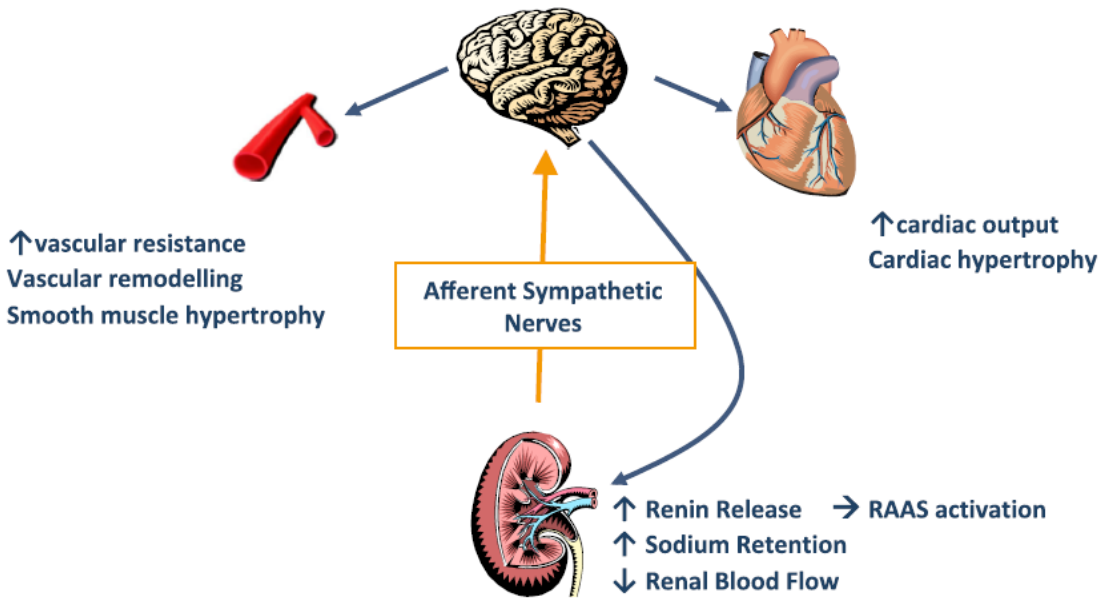

Figure 2: Schematic representing the relation between the SNS and renin-angiotensin-aldosterone system (35).

LHR accounts for about 25% of hypertensive cases and it is more commonly seen in older patients and in black more than in white patients (36, 37). LHR is marked by the presence of hypertension due to the low levels of plasma renin activity even following certain stimuli such as sodium restriction and the use of certain pharmacological agents, which in normal case would lead to an increase in plasma renin activity (38-40). Different causes for LRH have been reported such as aldosterone excess, mineralocorticoid excess, Liddle's syndrome etc.... For example subjects with an excessive production of aldosterone and where the system fails to reduce its levels, lower plasma renin activity were observed (41). It was suggested that the high aldosterone levels might be due to an increased adrenal sensitivity to angiotensinogen (42, 43). But not in all cases LHR are recognised to have high levels of aldosterone. LHR patients can have a normal level of aldosterone but on the other hand have high salt retention due an increased number of epithelial sodium channels (ENaC) thus increasing their extra-cellular fluid volume and eventually leading to the development of hypertension (44, 45).

c. Sodium, calcium and potassium balance

Ions are crucial for the proper functioning of cells and the maintenance of balanced action potential. Nowadays with the changes in the daily dietary supplies, the prevalence of hypertension has increased in the last two decades and one of the reasons being high sodium and a low potassium intake. All processed foods are rich in sodium and very low in potassium, on the contrary of natural food such as fruits and vegetables which are high in potassium but low in sodium (46, 47). Different studies

have showed the beneficial effect of potassium intake on cardiovascular health, for example Liu et al. showed that potassium reduced vascular damage and renal injury in hypertensive rats (48). In most of the cases, hypertension is accompanied by sodium retention. Sodium is reabsorbed in the renal tubules due to the activation of different pumps and co-transporters that are localised at the basolateral membrane and the luminal membrane respectively (47). The highest reabsorption of sodium occurs in the proximal tubule and thick ascending loop of Henle through the sodium-hydrogen exchanger type 3. This exchanger is observed to have an increased activity in hypertension (49). A low potassium concentration increases the activity of the sodium-hydrogen exchanger type 3, thereby rendering the intracellular compartment acidic and stimulating the SNS and RAAS (50). Hypokalemia exerts its negative effects not only on the kidney but also on the arterial wall. Low levels of potassium inhibit the sodium pump in the arterial and arteriolar vascular smooth-muscle cells thus increasing sodium concentration in the intracellular fluid. The increased concentration of sodium stimulates the sodium-calcium exchanger type 1 in the membrane driving calcium inwards. The inhibition of potassium channels and sodium pumps in the cells due to low levels of potassium induce a depolarization of cells in the smooth muscle of the vasculature. This depolarisation enhances the activity of the sodium-calcium exchanger, the voltage-dependent calcium channels in the membrane and the calcium channels in the sarcoplasmic reticulum thus increasing the calcium concentration in the cells and causing vascular smooth muscle cells to contract (51-53). Calcium can therefore be considered as a direct mediator upon blood pressure. It was shown that a higher

calcium intake induce an increase in sodium excretion thus reducing the intravascular volume and reducing the peripheral vascular resistance and blood pressure (54, 55).

d. Obesity, insulin resistance and hypertension

In the past few decades the prevalence of obesity has increased dramatically and it's causing important health burdens on the medical system due to its ensuing complications. In 2006, about 1 billion persons in the world were obese. According to the Canadian Health Measure 2009-2011, approximately 20% of young people aged between 12 and 17 are overweight and 10% are obese (56). Cardiovascular diseases and hypertension are two major risks that obese people encounter, the relative risk for cardiovascular complications increases with the increase in body weight (57). In obese subjects there is a need to increase the blood flow to tissues and organs due to increase workload and metabolic demands thus increasing cardiac output (58). Multiple mechanisms are altered in obesity; for example there is an increase in the SNS and an activation of RAAS. The exact mechanism that underlies obesity and hypertension is still not very clear. A study done by Wofford et al. (59) showed that if obese hypertensive patients were treated with α and β adrenergic blockers their blood pressure was lowered significantly more than in lean hypertensive subjects, thus showing the implication of the increased activity of the SNS. Increased RAAS activity in obesity is characterised by a high level of renin, angiotensinogen, angiotensin converting enzyme and aldosterone. In obesity, chronic activation of RAAS and the SNS results in increased sodium reabsorption and impaired natriuresis leading to volume expansion and hypertension (60, 61). Angiotensin is a very important factor in the determination of

insulin resistance in the case of hypertension. Angiotensin could interfere with the normal signalling pathway of insulin act on the serine phosphorylation of specific insulin receptors in the muscle cells thus contributing with the development of insulin resistance in essential hypertension (62, 63). In obesity, a chronic activation of RAAS and SNS are observed which leads to an augmented concentration of angiotensinogen and Ang II resulting in the long run in insulin resistance and hypertension.

3. Hypertensive treatment and impact on bone

There is a controversy on the impact of hypertensive medications on bone. Some studies show their beneficial effect on bone while others demonstrate their detrimental role. Some non-genetic aetiologies seem to be common between osteoporosis and hypertension; such as the low calcium intake and the low levels of vitamin D. Different treatments for hypertension are available some are based on physical activity, losing weight and reducing sodium intake and some other are based on the different classes of medications. Seven major classes of drugs are used to treat hypertension; they can be used alone or in combination dependent on the severity of hypertension. Anti-hypertensive medications target different molecules or pathways that underlie high blood pressure such as the diuretics which increase sodium excretion, ACE inhibitors, angiotensin receptor blockers, beta-blockers and calcium channel blockers.

a. Thiazide diuretics

Thiazides have long been known to be an anti-hypertensive drug that has a positive impact on bone. Thiazides inhibit the cotransporter of sodium and chlorine (SCCT) in the nephron and bone. SCCT play an important role in sodium and cholrine reabsorption in

the distal tubule. Thiazide enhances sodium urinary excretion and limits its reabsorption which leads to an increased urine fluid excretion, decreased fluid and plasma volume. All these changes will increase angiotensine, reduce cardiac output and finally reduce blood pressure. The reduced sodium reabsorption subsequently leads to a hypocalciuria thus increasing the availability of serum calcium. An increase in serum calcium levels inhibits the release of parathyroid hormone in the blood and therefore reduces bone metabolism (64).

b. Angiotensin-Converting Enzyme Inhibitors (ACE I)

As mentioned above, renin cleaves the inactive form of Ang I which is then cleaved by ACE and generates Ang II. The latter mediating multiple deleterious effects such as sodium retention, vasoconstriction etc... Blocking ACE will inhibit the production of Ang II thereby counteracting its negative effect and reduces blood pressure (65). It is believed that Ang II acts directly on the bone through the AT1 receptor that is present on osteoblasts and indirectly activates osteoclasts (66). It was also proposed by Grant et al. that Ang II also causes a drop in plasma calcium levels which stimulates the parathyroid hormone (PTH) secretion which indirectly activate osteoclasts and induce bone resorbtion (67). A large nationwide epidemiological registry survey showed that administration of ACEI was associated with a reduced risk of fractures by 7% highlighting the beneficial effect of this treatment on bone health (68).

c. Beta-blockers

The exact mechanism by which β-blockers reduce blood pressure is not fully understood. It is believed that it acts by reducing heart rate, cardiac output and plasma

volume. It also inhibits renin release from the juxtaglomerular cells through β1 receptor blockade. In a study carried by Reid et al. no consistent relationship was shown between treatment, bone mineral density and fractures (69). On the other hand, in another study, it was shown that β-blocker treatment was associated with 9% reduction of fracture risk (68). A meta-analysis of eight observational studies also showed the beneficial effect of β-blockers and its protective effect against hip fractures and any other fractures (70).

d. Calcium channel blockers (CCB)

Calcium is an important molecule for preserving many cellular processes in the body. Cellular function depends on the concentration of calcium inside and outside the cell, with the extracellular concentration being significantly higher than the intracellular compartment. The intra and extracellular concentrations are particularly important for the vascular smooth muscle cells. An influx of calcium through the calcium channels located on the cell membrane leads via complex mechanisms to cellular contraction. In hypertension, the disturbance in the sodium/calcium balance leads to this influx of calcium and to vascular contraction. There are different types of calcium channels and are targets of different subtypes of CCBs. The affinity of CCBs increases in the depolarization phase of the membrane, thereby interfering with calcium influx during depolarization (71, 72). Studies have not shown a substantial effect of CCB treatment on bone metabolism. Very few studies showed the protective effect of calcium antagonist on bone. In one of the studies done by Rejnmark et al., a 6% reduction in the risk of fractures was observed in patients treated with CCBs (68).

e. Loop diuretics

Loop diuretics are the most powerful natriuretic drug, however if they are not used in combination with another anti-hypertensive, loop diuretics are not useful for long term treatment. Loop diuretics primarily act on the thick ascending loop of henle by inhibiting the $Na^{+}/K^{+}/Cl^{2-}$ cotransporter. This inhibition reduces the transport of sodium chloride into the interstitial tissue. This class of medication reduces significantly blood pressure due to its natriuretic effect and the reduction in blood volume (73, 74). Loop diuretics have a negative impact on bone, since they increase calcium excretion in the kidneys thus increasing the levels of parathyroid hormone in the plasma. Different studies have shown the increased risk of fractures and most importantly hip fractures, in addition to a significant reduction of bone mineral density in the hip and entire body (75, 76).

f. Alpha-blockers

Alpha-Adrenergic blockers affect the α-adrenergic receptors and induce vasodilation. The α-blockers are not very popular due to their secondary effects for example they are known to induce postural hypotension especially in patients receiving concomitant treatment with diuretics (77). The impact of α-adrenergic blockers on bone is not very well known but it is hypothesized that because of its vasodilatory properties, patients experience faintness, postural hypotension thus increasing the risk if falls and fractures (78).

g. Angiotensin receptor blockers (ARB)

Following the activation of RAAS, Ang II reaches its final destination and binds to its receptors (AT1 and AT2). Different drugs have been developed to specifically target one

of those receptors. The most targeted receptor is AT1 considering its high prevalence in the kidney, heart, VSM cells, brain, adrenal glands, adipocytes and placenta. The activation of AT1 induces panoply of events including vasoconstriction and sodium retention which participates in the development of hypertension. Blocking this receptor by an Ang II antagonist reduces the RAAS effects and blood pressure (79). In animal studies, Ang II was reported to promote bone resorption via AT1 activation. Some studies showed the beneficial effect of AT1 and AT2 blockade on the increase of bone mass and attenuation of osteoclasts activity (64, 66, 80).

B. Osteoporosis

Osteoporosis is an age-related disease affecting both men and women but is more frequent among post-menopaused women. One in three women and one in five men over the age of 50 have osteoporosis. This musculoskeletal disease is one of the major growing health problems around the world and is related to the general aging of the society. The costs for treating osteoporosis and its related fractures are estimated to be 2.3 billion dollars as of 2010 in Canada alone (81). A major problem in osteoporosis is that it goes often undetected until bone fractures (82). The major risk factors in developing osteoporosis are hormone deficiency (oestrogen, testosterone), immobilisation, alcoholism, tobacco, low calcium intake and vitamin D deficiency. Osteoporosis is characterised by a low bone mass and defects in the micro-architecture of bone tissue, which leads to an increase risk of fractures due to fragility of the skeleton. The low bone density is generally caused by an imbalance in the bone formation and bone resorbtion process. Osteoporosis can be diagnosed by bone

densitometry, in addition to measurement of systemic parathyroid hormone, calcium and vitamin D levels. Osteoporosis is related to the deficiency of oestrogen in postmenopausal women and is generally related to the normal aging and occurs in both women and men.

1. Osteoporosis and bone remodelling

The skeleton of the human body is formed of 206 separate bones (83). The bones are the main reservoir of calcium in the body and they play an important role in protecting the vital organs such as the heart, lungs, bone marrow, brain etc... Although they look inert from the outside, bones are in a constant activity, they are in a continuous process of modeling and remodelling. There are two major types of bones: the cortical or also called the compact bone which has mechanical and protective functions and the trabecular (cancellous) bone which gives strength and provides the majority of the metabolic functions. Three types of cells exist in bones: osteoclasts, osteoblasts and osteocytes. The remodelling process mainly occurs in the trabecular bone and is therefore the site of diseases related to bone remodelling. Osteoclasts are giant multinucleated cells. They are bone resorbing cells and are responsible for secreting hydrochloric acid and proteolytic enzymes in the resorbing site. They are considered to be the main precursors of the remodelling process; their activity is regulated by local cytokines and systemic hormones (84). Osteoblasts are the cells responsible for bone formation during the modeling process. They form the unmineralised bone matrix such as type I collagen and non-collagenous proteins (e.g osteoclacine and osteonectin). They are also responsible for the active transport of calcium, phosphate and hydroxyl ions to

the extracellular space to form hydroxyapatite crystals. These crystals bind to matrix proteins lying in parallel with collagen fibers witch increase bone strength. The bone is composed of approximately 10% of cells, 60% of mineral crystals (crystalline hydroxyapatite) and 30% of organic matrix which is mainly constituted of collagen type I (88%)(85). In normal conditions, there is a balance between the processes of modelling and remodelling (Figure 3). This is a continuous physiological process in which old and damaged bones are removed by osteoclasts and are replaced by new bones formed by osteoblasts. An imbalance occurring during this bone activity will lead to the development of bone pathologies such as osteoporosis.

2. Modelling and remodelling of the bone

The modelling and remodelling occurs in 4 phases, during which osteoblasts and osteoclasts are monitored by different cytokines and hormones. During phase 1, osteocytes detect the changes and damages occurring in bones and initiate the remodelling process that involves the interaction of osteoclast precursor cells and osteoblast precursor cells. At phase 2, following the stimulation of hematopoietic stem cells in the bone marrow by monocyte/macrophage colony stimulating factor (M-CSF) and the receptor activator of nuclear factor kB (NF-kB) ligand “RANKL” (86, 87) they produce colony forming unit monocyte/macrophage lineage. Those mononuclear cells are considered to be osteoclast precursors and are attracted to the prospective resorption site, attach to the bone matrix and differentiate into active osteoclast. Mesenchymal stem cells (MSC) in the bone marrow produce osteoprogenitors which differentiate into pro-osteoblasts and then mature osteoblasts (88). Once the

osteoclasts attach to the bone matrix they form an isolated microenvironment. The zone of attachment between the osteoclasts membrane and the matrix is called the sealing zone, at this site we find the vaculolar H^+-adenosine triphosphatase (H^+-ATPase) also known as the "proton pump" (89). The bone resorption starts by dissolving the inorganic components of the matrix (e.g collagen type I) and then continue with the demineralisation of the matrix (90). The ion transport by the H^+-ATPase to the resorptive microenvironment renders the extracellular PH around 4.5 and this acidic milieu is responsible for mobilising the bone minerals. The demineralised organic components are then degraded by a lysosomal protease and by the cathepsin K (91). The products of bone degradation are endocytosed by osteoclasts and are transported and released at the cell's antiresorptive surface (92). The remodelling process enters phase 3 where oteoblasts take over and the bone formation begins. Osteoblasts will secrete alkaline phosphatase, collagen type I, proteoglycan, bone sialoprotein and osteopontin. They are found in clusters along the bone surface where they produce the bone matrix by collagen type I deposition and the consequent mineralisation of the matrix will be processed by the alkaline phosphatase (93). The bone formation continues even after the resorption phase, to ensure the proper balance between bone formation and resorption, and the remodelling process ends by bone mineralisation (phase 4).

3. Osteoporosis an age-related disease

Osteoporosis is an age-related disease also known as senile osteoporosis and is associated with aging in both women and men. Osteoporosis is due to the decrease in the bioavailable testosterone and oestrogen levels caused by aging and to other

environmental and medical conditions including reduced physical activity. More mechanisms like reduced calcium intake, reduced intestinal calcium absorption and impaired renal tubular calcium re-absorption due to ageing are important causes in bone loss. Age-related bone loss may be driven by gonadal sex steroid deficiency and hyperparathyroidism even at younger age. Decreased bone formation is generally attributed to decreased growth hormone (GH). GH is produced by the anterior pituitary gland; this hormone is responsible for the differentiation of marrow stromal cells (MSC) into osteoblasts and inhibits there differentiation into adipocytes (94). GH acts indirectly through stimulating the proliferation and function of osteoblasts through bone morphometric proteins (BMP) and Insulin growth factor 1 and 2 (IGF-1/IGF-2). IGF-1 is characterised for being GH-dependent, and is the primary mediator of the effect of the GH hormone. Therefore an increase in GH increases the expression of IGF-1 which will exert a primarily stimulatory effect on osteoblasts (95, 96). With age, the secretion of GH decreases and thus a decline in the level of IGF-1 will induce insufficient stimulatory signals to osteoblasts, thereby reducing the capacity of bone formation.

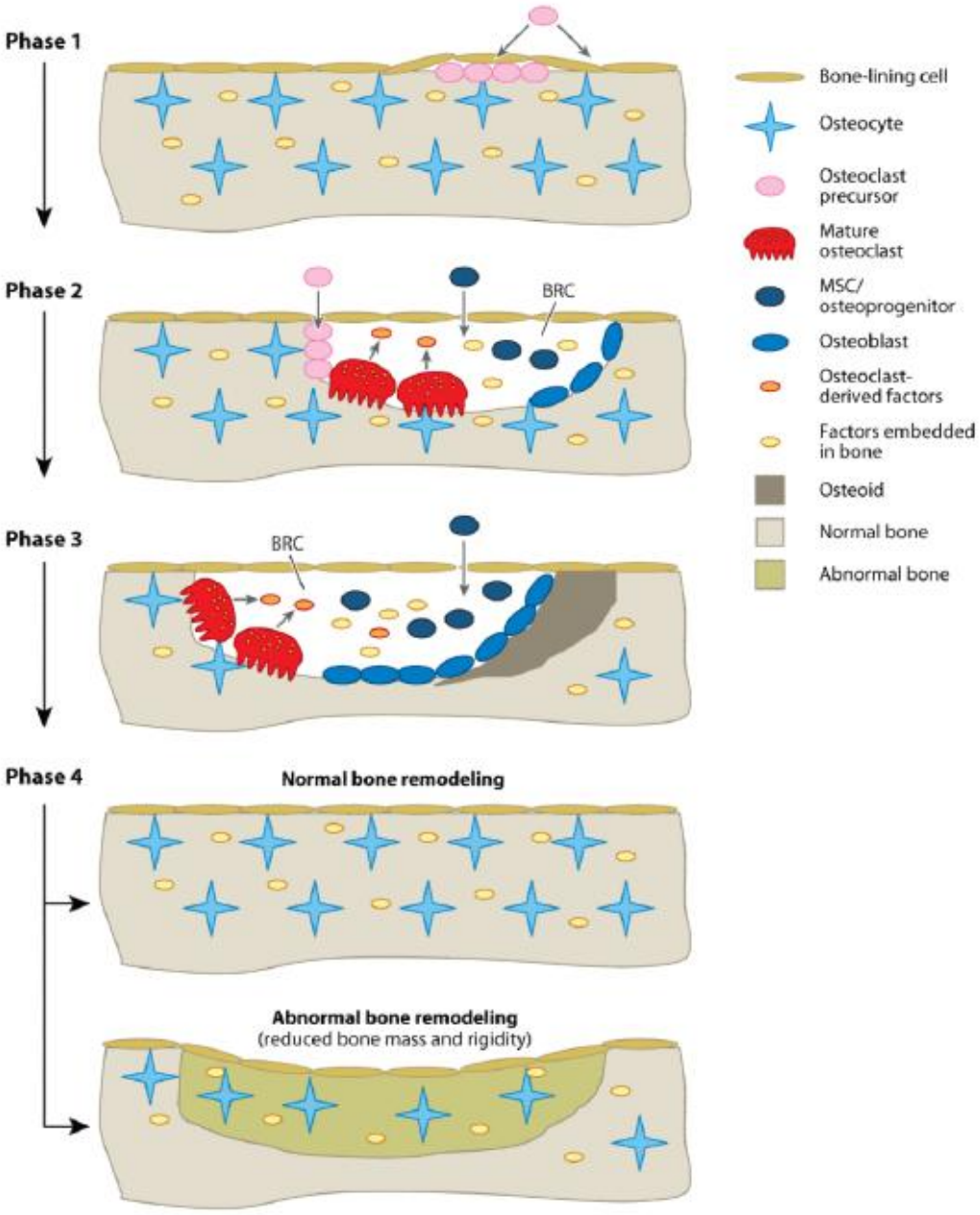

Figure 3: The bone remodelling occurs in 4 different phases: ***Phase1:*** Initiation/activation of bone remodelling at a specific site. ***Phase 2:*** Bone resorption and concurrent recruitment of osteoclasts and osteoprogenitors. ***Phase 3:*** Osteoblast differentiation (osteoid synthesis). ***Phase 4:*** Mineralization of osteoid and completion of bone remodelling (85).

A decrease in the IGF-1 level is associated with the aging process is therefore implicated in the pathogenesis of age-related osteoporosis. The fundamental role of IGF-1 in regulating bone formation is demonstrated by the analysis of IGF-1 deficient mice. This model showed skeletal malformations, delayed mineralisation, reduced chondrocytes formation and increased chondrocytes apoptosis (97). PTH is a hormone implicated in the regulation of calcium in the blood through binding to its receptor, the parathyroid hormone receptor. PTH increases the activity of 1-α hydroxylase enzyme which converts the 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol (the active form of vitamin D). The latter is responsible for the increased absorption of calcium in the intestine. Secondary hyperparathyroidism is characterised by an increased secretion of PTH due to hypocalcemia and due to a deficiency in vitamin D. Elderly people are generally deficient in vitamin D and calcium due to insufficient sun exposure, insufficient calcium intake and low physical activity etc... The Ultraviolet B from the solar radiation penetrates the skin and transforms the 7-dehyfrocholesterol to previtamin D3, which is then converted to vitamin D3 (98). In addition, it is shown that muscle strength is positively correlated with serum levels of vitamin D (99). Therefore, the lack of sun exposure and the reduced physical activity limits the production of vitamin D. The low level of vitamin D causes a reduction in intestinal absorption of calcium leading to a negative balance of circulating calcium thus increasing PTH levels in serum (100). PTH exists in high levels in serum of patients with osteoporosis and has also been implicated in age-related bone loss. The high level of PTH acts indirectly on osteoclasts. PTH stimulates osteoblasts which in turn will increase the production of RANKL. RANKL is

then able to bind to osteoclasts and stimulate their activation ultimately leading to bone resorption (101).

4. Vitamin D and calcium deficiency in osteoporosis

One of the main characteristics of osteoporosis is the deficiency in calcium and vitamin D. The principal ways for calcium absorption when it is found in high concentration in the lumen, is by passive diffusion through the intestinal membrane. A low concentration in calcium stimulates its active transport mediated by 1,25(OH)2D3. The latter is produced by the kidneys upon their stimulation by PTH, which is in turn dependant of the extracellular concentration of free calcium. The level of circulating calcium is sensed by the calcium sensing receptors located in parathyroid cell's membranes; therefore a low level of calcium would increase the PTH secretion hence the hyperparathyroidism observed in osteoporosis. Overall, the rate of calcium absorption is determined by the PTH-vitamin D-endocrine system (102). Oestrogen receptors are directly involved in calcium absorption, because of their receptors present in the mucosa along the alimentary tract, suggesting the important role of oestrogen in the intestine (103). In postmenopausal women and especially in women with osteoporosis, the level of circulating oestrogens is low which is associated with low calcium absorption and an increased bone resorption (104). The decrease in the vitamin D receptors (VDRs) is highly dependent on the levels of oestrogens. It is shown that oestrogen increases the number of VDRs in osteoblasts and intestine (105). So a deficiency in this hormone would lead to a decreased expression of VDR and a relative resistance to vitamin D. To this end, in the case of osteoporosis, we observe a low level of oestrogen accounting for

a low level of VDR and an increased resistance for the active form of vitamin D. Some experiments showed that VDR knockout mice developed severe hypocalcemia and an impaired bone mineralization (106). Furthermore, the oestrogen deficiency leads to an increased bone resorption which is evaluated by a loss of calcium. This low concentration of calcium stimulates the expression of PTH which in turn will increase the production of vitamin D. But the later will have no effect on calcium absorption due to the lack of VDR (104). Finally, the reduced circulating estrogen induces panoply of actions leading to impaired calcium absorption and an important contribution in the development of osteoporosis.

5. Osteoporosis and therapies

Different therapies are now available to treat osteoporosis and some are very well known for their capacity to increase bone mass. The treatments vary from daily calcium and vitamin D intake to administration of hormones, antibodies against one of the molecules implicated in bone resorption etc... The treatments that are available for all patients and have no or very low secondary effects are supplements of calcium and vitamin D, the dose prescribed usually is around 200-600 International Units of vitamin D per day and 0.8-1.2 grams of calcium per day, the doses certainly differ between the patients and is dependent on the severity of osteoporosis (107). In this regard MacKane et al. demonstrated that elderly women with secondary hyperparathyroidism started to have normal values of PTH in serum after 3 years of treatment with a calcium rich diet. Which proved that this hormone is directly associated with the calcium homeostasis (108). Teriparatide (Forteo), is a PTH analog it stimulates new bone formation by

activating osteoblasts, but this drug should be taken with a parallel administration of sufficient doses of calcium and vitamin D so it can exert its effect in restoring bone loss (109). The administration of calcintonin which is a hormone produced by the thyroid gland and counteracts the effect of PTH; it inhibits the activation of osteoclasts. The long term use of this hormone may down-regulate the calcitonin receptors on osteoclasts and may develop neutralizing antibodies, this treatment is effective in reducing bone pain but it doesn't seem to work on all patients (110). Denozumab is a fully human monoclonal antibody that binds to RANKL with high affinity and blocks the binding of RANKL to its receptor RANK; it mimics the effect of OPG. Denosumab (Prolia) is effective at a dose of 60mg subcutaneously every 6 months. Studies showed that this treatment results in a dose-dependent decrease in bone resorption (111), and is relatively well tolerated in patients. Raloxifene (Evista) is a new generation of oestrogen receptor modulator; it is the agonist of oestrogen and showed good preservation of bone density in postmenopausal women with osteoporosis. It acts as an agonist in bone and heart and as an antagonist in breast and uterus and possibly on brain. Raloxifen is also used for treating breast cancer. It was approved by the Food and Drug Administration in September 2007, but is now being reviewed by Health Canada. The use of Raloxifen in a clinical trial RUTH (Raloxifen Use for the Heart) including 10,101 women with a mean age of 67.5, with heart diseases, proved a decrease in breast cancer as well as a decrease in vertebral fractures. But Raloxifen treatment was also associated with an increase in fatal stroke incidence (absolute risk increase 0.7 per 1,000 woman-years) and venous thromboembolism (absolute risk increase of 1.2 per 1000 woman-years) (112).

Finally, the very important and the first line of use medication in treating osteoporosis for years is the Bisphosphanate family (Alendronate (Fosamax), Risedronate (Actonel) etc...). Bisphosphanate have a very high affinity for hydroxyapatite (the mineral component of the bone) and are very quickly cleared from the systemic circulation via their absorption by the mineralised bone. They are non-hydrolyzable pyrophosphate analogs. In high doses they inhibit mineralisation by interfering with the formation and aggregation of calcium phosphate crystals; they act as pyrophosphate by blocking the transformation of calcium phosphate into hydroxyapatite. But their clinical use in the treatment of osteoporosis is at low concentrations due to their inhibition of bone resorption by acting on osteoclasts. They induce osteoclastic apoptosis, inhibit osteoclast terminal differentiation and they decrease osteoclasts activity by inhibiting protein prenylation (113). They also act on osteoblast lineage thus increasing bone density on osteoporotic patients. Other studies showed that bisphosphanates modulate the expression of RANKL and OPG; they enhanced the production of OPG and reduced the production of RANKL (114). A recent publication showed that treatment with bisphosohanates in case of dental infections can cause osteonecrosis of the jaw thus causing severe problems leading to surgeries (115).

6. Diagnosis of osteoporosis

Different techniques and methods have been put in place for osteoporosis diagnosis. This age related disease is the major cause of fractures and its ensuing health burdens. Therefore screening for bone turnover markers, bone quality and BMD is very important for assessing osteoporosis and choosing the right treatment.

a. Bone turnover markers

The biomarkers that can be tested for in search of osteoporosis can be divided in two classes: bone formation markers and bone resorbtion markers. For the bone formation markers one can assess levels of osteocalcin, bone alkaline and type 1 procollagen-N-propeptide. The biomarkers for bone resorbtion are the following: type 1 collagen cross-linked N-telopeptide, type 1 collagen cross-linked C-telopeptide, tartrate-resistant acid phosphatase 5b and the undercaboxylated osteocalcin (116).

b. Dual Energy Absorptiometry (DXA)

DXA is the most popular imaging technique used in clinic for the diagnosis of osteoporosis and the follow up of treated osteoporotic patients. DXA provides the following measures: bone mineral content (BMC, in g), bone area (in cm2) and areal bone mineral density (aBMD, in g/cm2). DXA is usually used to assess BMD at the femoral site and at the lumbar spine; the aBMD at those sites of measurements is the best to predict fractures at those regions of interest. Some advantages and limitations exist for the use of DXA; the reproducibility of its results with a very low coefficient of variation, the general ease of use at different anatomical sites makes it very popular. On the other hand, the relatively high expenses and the difficulty in accessing this specialised machine in the health care system is a limiting factor (117).

c. Quantitative CT

Another technique that is less used in humans but widely used in research on animal models is the quantitative CT. This 3D imaging method measures volumetric density (in g/cm3) and it give the opportunity to measure cortical and trabecular BMD separately

on the contrary of the DXA that gives the combined aBMD. The different parameters that can be measured by this technique are bone volume, trabecular number, trabecular thickness, trabecular separation and the structural model index which represents the transition of the bone from a plate like shape to a rode like shape. In addition, cortic al thickness and porosity can also be determined (117).

d. Quantitative ultrasonography (QUS)

The principle of QUS is to use sounds that are lower (1kHZ) than the audible frequency threshold (20kHZ) (118). The mechanical and physical property of the bone and the surrounding tissues alter the shape, intensity and the speed of ultrasound that are emitted from the machine. Different measures are recorded that indicate the properties of the bone such as the broadband ultrasound attenuation (BUA, in dB/MHz), the speed of sound (SOS, in m/s) and the stiffness index(S.I) that is related to BMD (117). The QUS is mostly a technique applied on the calcaneus bone. This technique is attracting lots of interest in the clinical domain for its small size, portability and it's relatively low cost, in addition to the absence of radiation.

e. Bone mineral Density and T-score

T-Score,a surrogate of bone mineral density is used clinically to diagnose people with osteopenia and osteoporosis (119, 120). T-Score is defined as the standard deviation (S.D) of bone mineral density (g/cm2) from a reference healthy young adult population of women when using DXA. It is usually recommended that the reference population is calculated specifically for each study based on their representative sample population. The Lunar Achilles Insight/Express (GE Medical Systems Lunar, Madison, USA) is the

instrumentthat was used in CARTaGENE and it uses the QUS and provides a stiffness index (S.I) T-score. Stiffness index is primarily related to BMD and is calculated according to the following formula: SI= (0.67 * BUA + 0.28 * SOS) – 420. T-Score of S.I is the S.D from a sample of white women aged between 20 and 35 years (121, 122), T-Score = (S.I-mean of a young adult population)/S.D of mean young adult population. The most commonly used parameter to diagnose osteoporosis is T-Score and the following criteria apply: Above -1 is considered as "normal", from -1 to -2.5 is considered as "osteopenia" and below -2.5 as "osteoporosis" as defined by the WHO (119). The use of T-Score presents some limitations in terms of use in men, since it is calculated based on a young adult women population. Some considerations should be made to adjust the T-Score to a reference sample including men to give a more appropriate standard to the general population.

C. Recombinant inbred strain (RIS) of rats.

The polygenetic traits of hypertension make it difficult to determine its exact aetiology. The recombinant inbred strains of rats were created to identify the possible genes underlying this phenotype. The RIS is a unique set of rats that was originally created in the Institute of Biology, Faculty of Medicine, Charles University and the Institute of Physiology, Czech Academy of Sciences in Czech Republic. The RIS were first developed in the search of the different genetic loci that participate in the development of hypertension. The inbreeding was done from two genetically well-defined and very different strains of rats the Spontaneously Hypertensive Rats (SHR/Ola) and the Brown Norway rats (BN.Lx/Cub).

1. SHR/Ola

The SHR/Ola is an inbred strain that was developed by Okamoto and Aoki from Wistar rats that were bred under normal conditions. The rats that displayed the highest blood pressure for more than 2 months were chosen to produce the F1 generation. The average systolic blood pressure was between 150 and 175 mmHg for male and between 130 and 140 mmHg for females. From the F2 generation only rats with a blood pressure higher than 150 mmHg were chosen to continue the inbreeding and form the SHR strain. They observed that blood pressure increased with age and across the generations. The average systolic blood pressure for rats of 25 weeks of age was found to be 206±18.5 mmHg for males and 193±20.5 mmHg for females (123).

2. BN.Lx/cub

The inbred Brown Norway (BN) rat is a strain that was developed in the United States in 1917 from wild rats. Breeding pair were then transferred to the Institute of Biology at Charles University, Czech Republic and the BN were then called BN/cub. The inbreeding was carried to maintain their fixed genome. Parallel projects at the Institute aimed at developing an inbred strain of Wistar rats from an outbred strain of rats. The latter had developed a spontaneous mutation (Lx) predisposing the rats to the Polydactyly Luxate Syndrome (PLS). To do further genetic research concerning this syndrome, the trait was transferred on the genetic background of the BN/Cub hence forming the BN.Lx/Cub (124-126).

3. HXB/BXH (the recombinant inbred strain of rat)

The RIS also known as the HXB/BXH set of rats was created by reciprocal crossing of the Spontaneously Hypertensive Rats (SHR) (denoted H) and the Brown Norway rats (BN.lx) (denoted B). The HXB represents an SHR of female gender crossed with a male BN.Lx in a sort that all descendants would carry the mithochondrial DNA from the SHR and the Y chromosome from the BN.Lx; the crossing was made in the Czech Academy of Sciences. Whereas the BXH was developed at the Institute of Biology at Charles University, and the crossing consisted of having a BN.Lx as the female gender and the SHR as males thus transmitting the BN.Lx mitochondrial DNA and the Y chromosomes from the SHR. The cross of the two genetically well identified and very distant inbred parental strains, the SHR and the BN.lx generated the F1 generation. The inbred crossing of the F1 resulted in a unique F2 generation. The F2 is unique due to the genetic recombination of the independently inherited parental chromosomes found in the F1 generation. The problem in using the F2 is the inability to repeat the experiments with rats of the same genome considering its unique combination. Therefore, randomly chosen members from the F2 were brother-sister mated for more than 20 generations to eventually produce 36 RIS. The different recombinations during meiosis that occurred in the F1 helped in the spread of the parental genetic traits and the creation of unique members of the F2 generation. The advantage of using RI strains is their fixed genotype (within the same strain of RIS the genome is identical) thus facilitating longitudinal live long studies where animal of different ages share identical genotype within each RIS.

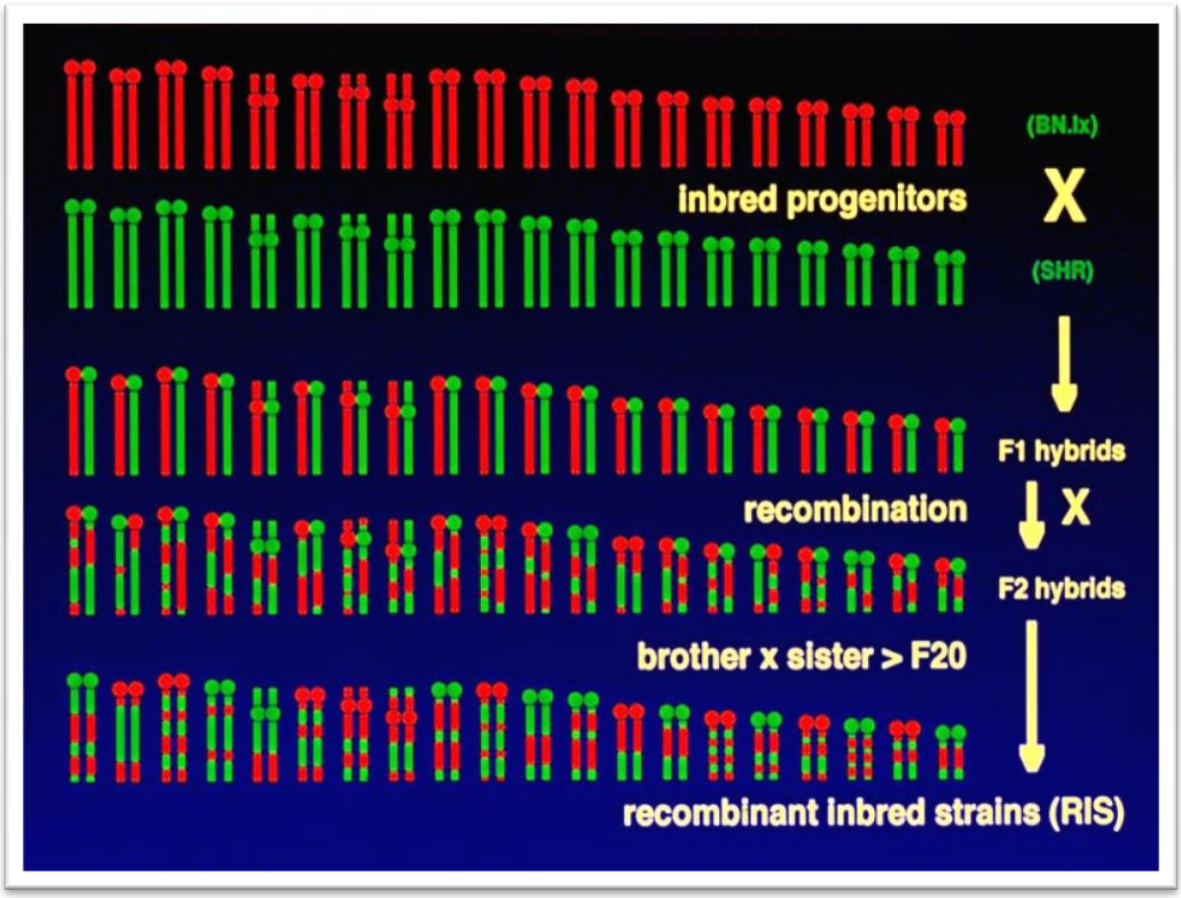

Figure 4: Schematic representing the crossing between SHR and BN.Lx that generated the recombinant inbred strains of rats. (Permission granted from Dr. P.Dumas copyrights©)

The well-defined genomic background of the parental strains helps in dissecting the little parts of the genome that segregated and that are responsible for the hypertensive trait (126, 127) (Figure 4).

4. RIS and blood pressure

The RIS make a good model for age-dependant physiological and genetic characterisation. Pravenec et al. monitored the blood pressure of 31 RIS by direct puncture of the carotid artery under light ether anaesthesia. They observed a continuous distribution of blood pressure that lied between the values of the parental strains (127). Twenty years later, Kunes et al. did the characterisation of the blood pressure for the same RIS using the same technique (carotid artery puncture) that was used 1989 in addition to the novel technique of the radiotelemetry to control for changes that might have occurred with time (128). Briefly radiotelemetry consists of inserting a telemetry probe in the abdominal cavity and its catheter in the abdominal aorta, the animals recover for 10 days after the surgery and blood pressure was monitored for 14 days. This technique became a standard nowadays because it allows the rat to be free in its cage and hence be able to record the blood pressure under normal activity and conditions (128). The results of this study showed a significant correlation between the old and new blood pressures. The radiotelemetry results were a little lower than the values of the direct carotid puncture and this is explained by the decreased stress of the procedure in the case of radiotelemetry. This study provides proof that the RIS are still a good model to study hypertension and that the use of telemetry probes for blood pressure monitoring is a relevant method to be used, and it

was further used for determining QTLs associated with blood pressure (127).This method is routinely used in our group and recently allowed us to establish important role of ENaC gene expression in hypertension using RIS as discussed bellow (129).

5. RIS and sodium balance

There are numerous mechanisms underlying the development of hypertension, one of them being the imbalance in sodium metabolism and storage. Several of genetic and physiological studies were carried to explore this phenomenon in the RIS set of rats. Studies showed an increased cation leak in red blood cells of SHR (130, 131). A study done by Talib et al. assessed the relation between high blood pressure and erythrocytes Na^+ content. Their results show that there is a significantly higher Na^+ leak from red blood cells and it significantly correlated with systolic blood pressure (SBP) in RIS and SHR (132). Initial studies from our group determined the kidney weight of SHR and RIS compared to the BN.Lx strain. SHR and RIS had a higher kidney weight at newborn and adult stage of life compared to the BN.lx, but the ratio of kidney size to the whole body weight was reduced in adult rats. Adult kidney weight was also seen to be negatively correlated with SBP at adult stage (133). The small kidney size compared to the total body weight suggests a smaller number of nephrons which threatens the ability of kidneys to excrete sodium especially if additional genetic or environmental factors are added and worsen the situation (134). This study also showed quantitative trait loci (QTL) significantly correlated with kidney size in newborns and adult rats, emphasizing the important role of genetic determinants in the pathophysiology of hypertension (133). Epithelial sodium channel (ENac) has been of interest to many to try and unveil its

link with hypertension. Recently a whole genome expression profiles of the kidneys of 3 different strains of recombinant inbreds and SHR their parental strain, showed an increase expression of mRNA for the genes (Scnn1b and Scnn1g) encoding ENac β and ɣ subunits. Those genes were also seen to be highly correlated with the systolic and diastolic blood pressure and the mean arterial pressure (129). In the above mentioned studies from our group, it was shown that sodium equilibrium and its genetic component are very important parameters in the development of hypertension.

6. RIS, gene and environment

The use of RIS in studies of hypertension is facilitated by large number of strains with a continuous distribution of blood pressure, signature of its polygenic character. Their well-defined genetic background and their physiological traits for hypertension make them a seminal rodent model to study. A study done by Dumas et al. demonstrated the influence of the environment and the genetic components on ions excretion in the recombinant inbreds. It is hold that the excretion of sodium is the equivalent of sodium intake (135), implying the importance of the environment on this physiological aspect. In order to discern the difference between the environment and the genes, it was shown that fasting state reduces environmental variability. The entire set of RIS along with the parental strains (BN.Lx and SHR) had their levels of Na^+, K^+ and Ca^{2+} measured, a strain distribution pattern (SDP) was drawn and heritability was calculated. Under free access to food the environmental component (calculated as a sum of intra-strain variances within RIS) was responsible for 71% of the variance of Na^+ excretion and only 29% was genetic. When the rats were subjected to 26 hours fasting (zero sodium

intake), they showed immediate salt retention in SHR and graded salt retention/loss in RIS a strong genetic heritability was demonstrated (70% of the variance) whereas only 30% was environmental. Similar results were shown for K^+ and Ca^{2+} excretion. In addition, genetic association studies, using the excreted ions as a phenotype revealed a set of genetic markers pertinent to ions handling in context to blood pressure modulation (136). Another study done by our group showed the impact of the environment on genetic modulation. In search of the genes responsible for stress response in hypertensive rats, different RIS and their parental strains had a continuum of response to stress as shown by their strain distribution pattern of body temperature, again a polygenic phenotype. Hypertensive rats had a higher rise in temperature and blood pressure following 20 minutes of immobilising stress than the normotensives. This increase in body temperature was associated with 2 interacting genetic loci. In addition, under high sodium diet the response to stress was greater in hypertensive rats (137).

7. RIS and insulin resistance

Hypertension is frequently accompanied by other cardiovascular risk factors such as dyslipidemia, diabetes and insulin resistance, the latter being proposed as one of pathogenetic mechanisms of high blood pressure. SHR is known to be a good model for hypertension and other metabolic derangements (138, 139). Insulin resistance was one of the features seen in SHR besides hypertension. Different physiological and genetic analyses were done to try and link the coexistence of these two diseases. In a genetic study using the RIS and the parental strains (BN.Lx and SHR) done by Aitman et al. it was shown that insulin resistance was mapped on two chromosomic locations (Chromosome

4 and 12) (140). Later on, the same group showed that one of the genes underlying insulin resistance is the cd36 gene. The latter is a gene located on chromosome 4 that encodes a transmembrane fatty acid transporter. In SHR they observed a deficiency in cd36, which explains the perturbation in fatty acid transport in adipose and muscle tissues, the impaired insulin activity and glucose intolerance (141). Genes modulating blood pressure were previously shown to map on chromosomes 1-2-4-3-13 and 20 (127, 142-145). Chromosome 4 is the common chromosome on which genes of blood pressure and insulin resistance exist, which could explain the concomitant manifestation of hypertension and insulin resistance. We are reporting in this thesis that longitudinal observation of insulin resistance seem to be subsequent to hypertension rather than preceding it in RIS (manuscript 2).

D. Hypertension and accelerated ageing

Properties of accelerated ageing such as increased cellular turnover, increased apoptosis, cardiac and renal hyperplasia were demonstrated to be characteristics of hypertensive animal models such as the SHR. Our group performed series of studies addressing physiological and genetic mechanisms underlying specific hypertension related traits. Hadrava et al. did a study on the aortic smooth muscle cells ex-vivo from hypertensive and normotensive rats to determine their proliferative ability and efficiency. Cells from SHR had a significantly higher growth rate than the Wistar Kyoto (WKY) rats implying genetic determinants behind this feature considering the absence of high blood pressure in cellular culture. In addition the rate of growth-decline after reaching cellular confluence in vitro was slower in SHR compared to WKY indicating a

enhanced response to proliferative stimuli in SHR (146). Another feature of accelerated ageing is increased cellular apoptosis, which is remarkably higher in hypertensives compared to normotensives. A study done on SHR, WKY and BN, showed an increased level of apoptosis in target organs of hypertension such as kidneys, heart and the brain in SHR compared to their normotensives control the WKY and BN (147). In the same context a study carried on WKY and SHR showed a higher cellular turnover in the kidneys, heart and aorta in SHR compared to WKY. The half-life of cells from those organs was decreased of about 50% in SHR, which is seconded by a shorter cell cycle in this hypertensive strain. They also showed that the telomere length of genetically hypertensive rats was shorter than the normotensives (148). Investigation in humans, done on pairs of twins from the Danish Twin Register showed a significant negative correlation between pulse pressure and telomere length (149). Integrative information from the above mentioned studies support the notion of hypertension as a form of accelerated ageing.

E. Hypertension, calcium metabolism and bone

1. In the animal model

The SHR is the best animal model that displays essential hypertension and serves as a good tool to study abnormal calcium metabolism and its implication on hypertension and bone loss. McCarron et al. first investigated the calcium metabolism in SHR compared to their normotensives control the WKY rats, and observed that the SHR displayed a low concentration of ionised serum calcium, an increased calcium excretion (at 17 weeks of age) and an increased parathyroid hormone levels in the blood

compared to WKY (150). Later on, another group studied the impact of high calcium diet on young SHR (6 weeks old) compared to WKY. They observed that the increased serum calcium reduces natriuresis which participates in lowering of blood pressure in SHR. But they did not see any changes in the level of PTH nor calcium excretion which could be due to the age factor (151). Few years after those two studies, Izawa et al. tackled the issue of calcium, hypertension and bone mineral density in SHR. They compared the mean cortical thickness and the trabecular bone in the tibia of the SHR to WKY rats and observed that SHR displayed a significantly lower bone volume, which suggests the development of osteoporosis in the SHR (10). A study done by Lucas et al. on SHR and WKY observed a low level of vitamin D3 (1,25(OH)2D3) in SHR compared to WKY. In addition, SHR of age 20-30 weeks displayed a significantly lower bone mineral density and calcium content compared to WKY suggesting a form of bone disorder (152). Investigation the effect of antihypertensive drugs on bone loss in stroke prone-SHR models indicated the positive effect of amlodipine on bone through direct inhibition of osteoclasts (153). A review by Cappuccio et al. discusses the role of kidney and kidney stones and their link with hypertension and osteoporosis. Hypertensive subject and SHR models are more prone to develop kidney stones due to their increased calcium excretion. This increased calcium excretion will eventually cause an increase in bone resorption as a compensatory mechanism and therefore cause bone loss and osteoporosis (Figure 5) (154).

2. In humans

Explorations in human populations investigated links between calcium, bone loss, hypertension and other cardiovascular diseases. Hvarfner et al. studied calcium metabolism in 182 subjects which included 58 participants with essential hypertension. The latter were found to have an increased urinary calcium excretion, an increased level of PTH and a reduced level of ionised calcium compared to their normotensives controls. The concentration of urinary calcium and plasma PTH were positively correlated with blood pressure, whereas serum calcium was inversely correlated with it. Their hypothesis of impaired renal calcium reabsoprtion in essential hypertensive subjects was supported in this study, thus explaining the reduced plasma calcium and the resulting increased PTH levels (155). A study done on 3676 white women with an average age of 73 years, in whom blood pressure and BMD were measured. It was found that the systolic blood pressure is significantly negatively correlated with bone mass (implying an increased calcium loss) at the femoral neck (13). In another study, conducted in 82 women (average age of 59 y/o) with mild to moderate hypertension and 40 postmenopausal women as control; blood samples were drawn and DXA was performed on the lumbar spine (L2-L4). Different markers of the bone were measured such calcium, phosphate, PTH, vitamin D and osteocalcin. Their study demonstrates an increased calcium excretion in hypertensive women compared to normotensives in addition to an increase in osteocalcin levels.

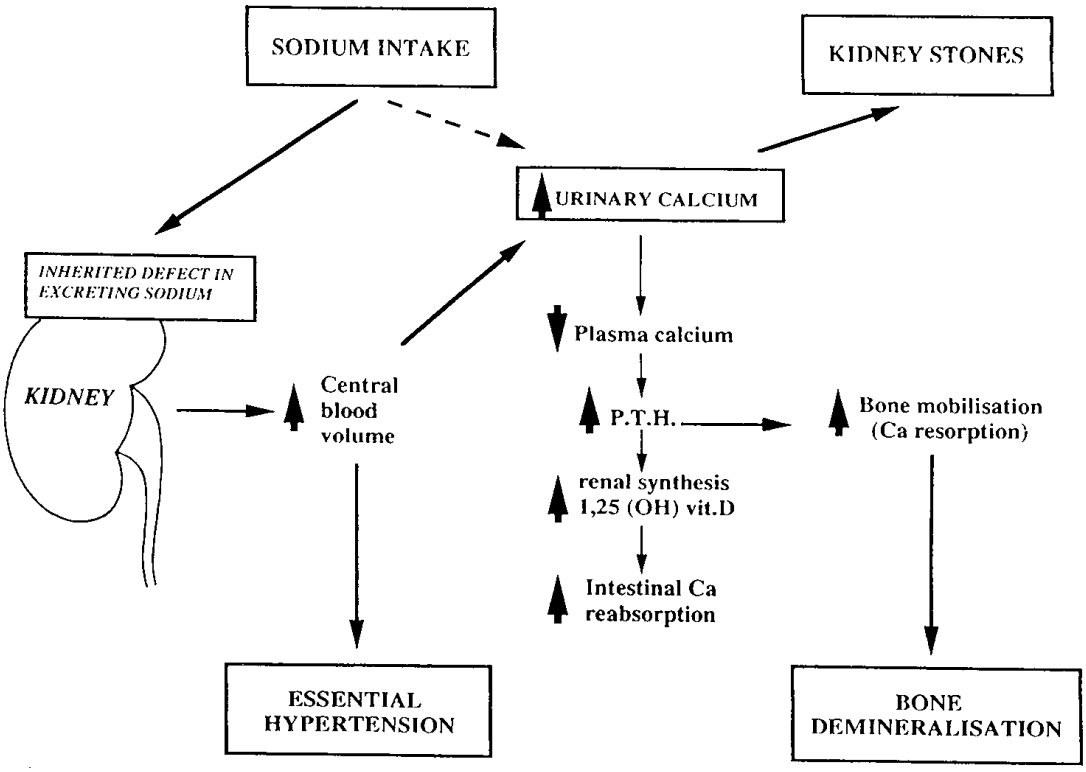

Figure 5: A schematic representing the link between calcium, sodium and vitamin D equilibrium and the role of kidney in the pathophysiology of hypertension and their implication on the bone disorder (154).

Osteocalcin, a bone turnover marker and calciuria were observed to inversely correlate with blood pressure, but they did not find a direct relation between bone mass and hypertension (156). Another type calcium metabolism alteration was reported by Brickman et al. In their study they investigated the relation of plasma ionised calcium, blood pressure, PTH, vitamin D and intracellular platelet calcium concentrations. As reported in previous studies, levels of calcium were seen to be decreased in the plasma whereas levels of PTH and vitamin D were increased. Interestingly in this study, intracellular concentration of calcium in platelets was observed to be increased in comparison to normotensives subjects. There was a positive association between PTH, vitamin D, intracellular calcium in platelets and blood pressure and an inverse relation was seen among ionised plasma calcium and blood pressure. The difference between intracellular and extracellular calcium observed in hypertensive subjects indicates the implication of factors altering compartmentalisation of calcium (157). All those studies emphasize the abnormal calcium metabolism observed in hypertensive patients and also in animal models of genetic hypertension. The exact causes and pathway are still not entirely clear, but it is evident that this impairment in calcium handling is affecting bone health and thus increasing the chances of osteoporosis and hypertension to coexist.

F. Hypothesis

Project 1

Association of age-dependent height and bone mineral density decline with increased arterial stiffness and rate of fractures in hypertensive subjects.

In this project we tested our hypothesis that hypertension is a form of accelerated ageing in a population based cohort, CARTaGENE executed in Quebec in 2010-2011. The strength of this cohort is the availability large set of determinants measurements such as bone mineral density, blood pressure and arterial stiffness but also health status, consumption of medication, habits etc. Our first observations started in the Canadian Heart Health Study and in the Saguenay Lac Saint Jean population, where we observed a shorter stature of hypertensives compared to their normotensives controls in older subjects, contrasting with taller height in young hypertensives. In CARTaGENE, we validated our previous observations and assessed the relation between height, blood pressure and bone mineral density. We then explored the relation between height, hypertension, arterial stiffness, bone mineral density and fractures.

We hypothesised that hypertensive subjects would display a shorter stature in elderly, a higher rate of fractures and low BMD. In addition, we tested whether arterial stiffness could be the best cardiovascular parameter that is associated with height, low BMD and fractures.

Project 2

Temporal aspects of development of hypertension and low bone volume in SHR and recombinant inbred strains of rats.

In this project we tested the hypothesis of hypertension and osteoporosis as putative forms of accelerated ageing sharing common genetic determinants. We therefore used three different strains of recombinant inbreds and one of their parental strains the SHR. The strains used in this study are the following HXB/BXH set with HXB (H representing a female SHR and B representing a male BN.LX) and BXH (H representing a female SHR and B representing a male BN.LX): HXB 3, HXB13, HXB17, HXB26, HXB29, BXH2, BXH5, BXH9, BXH11 and BXH13. Our objective was to follow those rats for a period of one year and assess the development of different phenotypes such as hypertension, low bone mineral density and insulin resistance. The onset of hypertension in those strains is known to be in their very early stages of life, yet to our knowledge no one studied, the bone morphology of those animals along with the development of hypertension. We therefore assessed their bone microarchitecture using a 2D and 3D imaging technique and analysed their bone properties (trabecular number, bone volume etc.). The defined phenotypes were measured at four stages of their life (3, 6, 9 and 12 month) in order to determine the onset of age-related abnormalities. We then carried linkage analysis in order to identify possible common genetic determinants underlying hypertension and low bone density. ***We hypothesised that the RIS develop low bone mineral density along with blood pressure and could be sharing common genetic determinants between the two phenotypes.***

Results

Manuscript 1 (submitted June 2014)

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Journal of Hypertension

Association of age-dependent height and bone mineral density decline with increased arterial stiffness and rate of fractures in hypertensive subjects.

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Abstract

Background: Hypertension and osteoporosis are age-related health risks differentially expressed in men and women. Here we have analyzed their prevalence in a randomly selected cross sectional cohort (CARTaGENE) of Quebec, Canada and explored their existing relationships along with height, arterial stiffness and bone fractures. **Methods:** CARTaGENE includes 20,007 subjects, of 40 to 70 years of age. Participants were subjected to an extensive phenotyping and a questionnaire of past medical history and habits. **Findings:** We determined the differences in height of participants and their relation to hypertension status and sex in this cohort and validated it in two other cohorts (The Canadian Heart Health Study and a family cohort from the Saguenay Lac Saint-Jean, a region of Quebec). In all three cohorts, we found that at younger age hypertensives are taller than normotensives but they have a shorter stature at older age compared to normotensives. In CARTaGENE, we observed that both hypertension and arterial stiffness are strongly associated with height when adjusted for antihypertensive medication ($p<0.0001$). This observation led us to hypothesis that height loss may be due to a loss in BMD. We therefore investigated the relation between height and the broadband ultrasound attenuation (BUA) and we observed a significant association in men (age group 40-54) and women (all ages). This prompted us to investigate the possible relation between the two ageing processes: hypertension and low BMD. Fractures are the net outcome of low BMD and a significant association is observed (OR=2.34, CI=2.12-2.57); this relation was stronger in hypertensives compared to

normotensives. In addition, we observed that increased arterial stiffness was significantly correlated with a lower BUA in both men and women at all ages. **Interpretation:** Shorter stature in elderly and low BMD and fractures correlated with increased arterial stiffness in hypertensive subjects. We propose that hypertension and osteoporosis share components of accelerated aging.

Funding: The Canadian Institute for Health and Research (CIHR),Genome Quebec,Genome Canada and Canadian Partnership for Tomorrow Project (CPTP).

Keywords: Hypertension, height, arterial stiffness, bone mineral density, fractures.

Introduction

Hypertension and osteoporosis are two age-related diseases that are major health burdens in Canada and worldwide. We previously proposed hypertension a form of accelerated ageing characterized by accelerated cellular turnover and faster shortening of telomere length in genetically hypertensive rats.[1] Different studies showed that hypertension is a form of accelerated growth, maturation or aging with metabolic changes and hypertrophy of the arterial wall smooth muscle cells associated with accelerated biological maturation, leading to high blood pressure and cardiovascular outcomes.[2] In addition, it was suggested from a small cohort of ~100 individuals that hypertensive children have an accelerated skeletal growth compared to their healthy controls.[3] McCarron et al. reported in 1980, that hypertension is associated with abnormal calcium metabolism, including an increase in calcium excretion for a given sodium intake, and an increase in parathyroid gland activity.[4] In the last decade, many studies tried to unveil the existing relation between hypertension and osteoporosis in animal models and in humans.[5, 6] The direct association between these two diseases in a large population-based study has not yet been reported. However, a small study by Tsuda et al. on 31 hypertensive and 14 normotensive Japanese women showed that the bone mineral density (BMD) in the lumbar spine was inversely correlated with systolic blood pressure. In addition, the 24h urinary calcium excretion was increased compared to normotensives.[7] In a large case-control study, Vestergaard et al. assessed the impact of hypertension and other cardiovascular diseases on the risk of fractures and observed that hypertension and stroke were important independent cardiovascular risk factors

for fractures.[8] Andreassen et al. recently identified common genetic variants of blood pressure that are associated with other phenotypes. Among the significant single nucleotide polymorphisms that were associated with systolic blood pressure were those associated with bone mineral density and height.[9] In previous studies that we conducted with the Canadian Heart Health study and the family cohort of the Saguenay Lac-St Jean area, we noticed a difference in body height between hypertensive and normotensive subjects.[10] Our current analysis was performed in a population-based cohort, "CARTaGENE". It represents a randomly selected sample of Quebec's population and included 9,628 men and 10,261 women. Our objective was to explore the notion that hypertension is a form of accelerated ageing and to search for the potential relation between four ageing processes: hypertension, arterial stiffness, short stature and low BMD resulting in fractures.

Methods

Subjects

CARTaGENE is a population based cohort established in 2003 in Quebec (Canada). It includes 20,007 randomly selected subjects aged between 40 and 70 years. Available data analysed here is from 19,889 subjects including 9,628 men and 10,261 women evaluated from August 2009 to October 2010. The recruitment of subjects and study design of CARTaGENE were recently described.[11] Ethics approval was granted by the Faculty of Medicine of the University of Montreal and the Ethical research committee of the CHUM Research Center.

Data from two other cohorts were used to study the relation between hypertension status and height analyses: the population health survey Canadian Heart Health Study and 120 families of the Saguenay-Lac St-Jean population of Quebec (French Canadians).

- Canadian Heart Health Survey (CHHS) is a study that included patients from 10 Canadian provinces from 1986 till 1992. A total of 23,129 subjects from 18 to 74 years old participated in the study: body height, blood pressure and weight were taken among other physical measurements.[12]
- 120 extended families from the Saguenay-Lac St-Jean population (SLSJ) of Quebec which reside in a relatively isolated region and the genealogical records dating to the 17th-century are available. Those families were ascertained by the presence of a sibpair affected with early-onset of hypertension and/or dyslipidemia. Anthropometric measurements including body height from 849 participants' (older than 18 years) were used in the current analysis.[10]

Health questionnaire

CARTaGENE used a standardized validated interviewer-based questionnaire, in addition to a self-administered questionnaire answered by all participants. The interviewer-based questionnaire covered health and medical history and included a detailed history of diagnosis of hypertension and osteoporosis among other diseases. The prescribed medications administered to the participants were all recorded from the packaging or from the medicinal products themselves, in addition to all other supplements taken without prescription.

Procedures:

All procedures were performed according to the standard operating manual either as previously reported by Kotchen et al.[13] or as described by Awadalla et al.[11]

Peripheral BP

Peripheral BP was measured using an Omron IntelliSense BP Monitor (HEM-907XL). Appropriate cuff size was applied to the non-dominant arm, with the participant in a sitting position. The readings started 10 min after being seated and three measurements were taken 5 min apart. The mean of all available BP measures was used. Hypertension was defined as a mean systolic blood pressure (SBP) ≥140 mmHg, and/or a mean diastolic blood pressure (DBP) ≥ 90 mmHg, and/or being treated for hypertension.[14]

Applanation tonometry

After peripheral blood pressure measurement, the same arm was used for applanation tonometer recording. A micromanometer-tipped probe (SphygmoCor™, AtCor Medical Pty Ltd, Australia) was applied at the surface of the skin overlying the radial artery and a continuous recording of the peripheral radial pulse wave form was carried for 5 minutes. The methodological details of this measurement were described by Siebenhofer et al.[15] Augmentation index (Aix) in percentage, an indirect measure of arterial stiffness, was calculated as follows: (Augmentation Pressure/ Pulse pressure) * 100.[16]

Bone Mineral Density (BMD)

BMD was measured on the calcaneus bone using the Lunar Achilles Insight/Express (GE Medical Systems Lunar, Madison, USA). The Achilles Quantitative Ultrasound System measures the speed of sound and the frequency-dependent Broadband Ultrasound

Attenuation (BUA) and combines them to form a clinical measure. The low BMD group included the osteopenic and osteoporotic categories and those subjects taking osteoporosis medications.

Height

Height was measured without shoes, using the Seca 214 Stadiometer (graduation: 1mm). Three repeated measures were taken and their average was used in the current study.

Statistical analysis

The analyses were performed using SAS 9·2. Eighteen thousand three hundred forty seven (18,347) participants had non-missing data for bone and hypertension phenotype analysis, whereas thirteen-thousand four hundred seventy one (13,471) participants had interpretable non-missing data for arterial stiffness. Descriptive statistics such as numbers, proportions for categorical variables, means and standard error of the mean for continuous variables were used. Analysis-of-variance (ANOVA) was used for continuous variables. Logistic regressions were used for categorical variables and Odds Ratio (OR) was calculated. Simple linear regression was used to assess the association between continuous variables. The significance threshold was fixed at 0.05.

Results

Body height

31·8 % of CARTaGENEs' participants were hypertensives; the prevalence of hypertension was higher in men (57·1 %) than in women (42·9 %) (*Table 1)*. We investigated the differences in height between hypertensives and normotensives in the Canadian Heart

Health Study and observed that hypertensives were taller before the age of 50 and had a shorter stature after that age compared to normotensives in both men and women; the slope of decline was significantly steeper in hypertensives compared to normotensives (slope difference in men p=0·0032; slope difference in women p=0·0012). Another validation analysis was performed in the family cohort of the Saguenay-Lac St-Jean population of Quebec.[10] The analysis showed that hypertensives were taller before the age of 36 compared to their normotensive siblings, and were shorter after that age. This relation was true in both sexes (slope difference in men p=0·0175; slope difference in women p=0·0321). We then performed the same analysis in CARTaGENE, which includes only subjects over 40 years old, and observed that older hypertensive men were shorter than normotensives (slope difference p=0·0008). Whereas hypertensive women were shorter than normotensives already in the group of 40 to 54 (the slopes were not significantly different), significantly shorter (by 1cm) in both age groups (p<0·0001) (*Figure 1* and *Table 2)*.

Bone and arterial parameters

In a second step, we investigated the relation between height, BUA and other blood pressure parameters including arterial stiffness in the CARTaGENE study. Our association analysis showed that among the cardiovascular parameters, the augmentation index (a surrogate of arterial stiffness) had the most significant association with height (p<0·0001) and it contributed to 20% of height changes (R^2= 0·20) *(data not shown)*. This association was found to be true in both men and women at all ages (*Table 3)*. We then considered whether the decrease in body height is due to

bone mineral density loss. The association between BUA and height was significant (p<0·0001) and contributed to 4% of height changes observed (R^2= 0·04) *(data not shown)*. BUA and height were significantly correlated in younger men (age group 40-54) and in women (all age groups) (*Table 3)*.

Association of hypertension and bone fractures

The association of height with hypertension on one hand and with low BMD on the other hand led us to assess the possible relationship between hypertension and low BMD. We therefore examined the prevalence of low BMD among hypertensive and normotensive subjects. We observed that 17·1% and 12·1% of normotensives and 20·8% and 15·3% of hypertensives (P<0.0001) had a low BMD and fractures respectively (*Table 1).* The higher rate of low BMD and fractures persist despite the significantly higher osteoporotic treatment received by hypertensives compared to normotensives (P<0·0001). Bone fracture has always been known to be the result of a low BMD, in CARTaGENE we observed a significant relation between low BMD and fractures (OR=2·34, CI=2·12-2·57) *(data not shown)*. This relation was increased in hypertensives compared to normotensives in both sexes and at all ages (*Table 4)*. We then analyzed available peripheral and central parameters of blood pressure and assessed their potential association with BUA. We observed that Aix, central and peripheral pulse pressure (cPP and PP) respectively were inversely associated with BUA (P<0·0001), indicating a decreased bone mineral density with the increase in central and peripheral blood pressure parameters (*Table 5)*. The relation between arterial stiffness and BUA

remains even after stratification for age and sex; with risk of fracture more pronounced among hypertensives of both sexes, particularly at younger age of 40 to 54 ***(****Table 6)*.

Discussion

CARTaGENE study is both a population-based biobank and the largest ongoing prospective health study of men and women in Quebec (Canada). It is a population based cohort that has a very comprehensive information database concerning the health of its participants. The importance of this cohort resides in the presence of physical measurements data, including parameters such as bone mineral density and arterial stiffness. In 1990, the prevalence of hypertension in Quebec's population was 20·8% [17] and rose to 31·8% ten years later (from CARTaGENE's data). In Canada, the prevalence of hypertension was 22% in 1997 [12] and was reported to be 19% ten years later (the Canadian Health Measures Survey 2007-2009).[18] This high prevalence in Quebec in the last decade could be explained by different environmental components such as lifestyle, dietary intake, obesity and other factors. In the Canadian Health Measures Survey, the blood pressure determination was done using the BpTRU device where the first reading was omitted. Since the first measurement is generally higher in this procedure, the decrease in the estimate of hypertensives in Canada observed in the 2007-2009 period could have been overestimated, and the real prevalence not diminished.[19]

We previously suggested that hypertension is a form of accelerated ageing.[1] Observations concerning the height of hypertensive subjects compared to normotensives, from two previous studies in which we participated (CHHS and SLSJ) led

us to hypothesis of the potential relation between hypertension and height, two traits affected by ageing. We therefore investigated this relation in all three cohorts. In the CHHS and SLSJ we found that hypertensives are taller than normotensives at a younger age, but display a lower body height in elderly, suggesting a more rapid decrease of height as compared to normotensives. A genetic finding that could explain this height difference is the single nucleotide polymorphism rs1874952 that resides within the potassium voltage-gated channel gene (*KCNAB1*) that was found to be linked and associated with adult height in hypertensives only (http://www.pulsus.com/ccc2010/abs/065.htm). This channel is present in osteoclasts and can regulate their activity. In addition, *KCNAB1* gene was reported to contribute to the osseographic scoring system (OSS) in the Framingham study.[20] In CARTaGENE, we observed a shorter stature of elderly hypertensives compared to elderly normotensives. This incited us to question if this could be due to bone loss, and our analysis confirmed a significant association of height and BUA decline with age, more pronounced in hypertensives. A common fact that is observed in the aging population worldwide is height decline with age. Height measurements are usually used to assess the socio-economic status of a population, for example childhood nutrition status and the disease environment etc.[21] Rare are the studies that unveil the relation between height decline and bone loss. A study performed by Galloway et al. on 1,024 subjects (735 women and 289 men) evaluated the correlation between height decline and bone loss with ageing. Their findings show that bone mineral density (BMD) plays the largest role in determining annual height reduction.[22]

We then studied the relation between hypertension, bone mineral density and fractures and observed a higher rate of hypertensive subjects with low BMD and fractures compared to normotensives. Results of other different studies are in agreement with our observations, such as the cross-sectional analysis published by Wada et al. [23] on Japanese women that showed a significantly higher prevalence of hypertension in women with vertebral fractures compared to those without fractures. A study done by Sennerby et al. on 31,936 Swedish twins showed that diagnosis of cardiovascular diseases (CVD) was significantly associated with an increased risk of hip fractures. This suggests the presence of genetic determinants underlining CVD and the high risk of hip fractures.[24] In a recent publication that covered many different experimental and clinical studies, it was suggested that CVD and osteoporosis are significantly associated.[25] In addition, Andreassen et al. observed a set of common SNP's between blood pressure and BMD for the *IKBKAP*, *NMT1* and *PLCD3* genes which could represent the link between those two phenotypes.[9] Different metabolic disorders such as calcium handling that are seen in these two diseases may explain pathophysiological aspects of the link.[7] Despite the accumulating evidence of the link between these two age-related diseases, no official guidelines were set to be put in clinical practice. The rates of low BMD and fractures often increase with age, in our data in the age group of 40-54 there was 11·9% and 9% of low BMD and fractures compared to 27·3% and 18·8% respectively in the older age group (55-70). The increased rate of low BMD and fractures was more pronounced in younger hypertensive subjects, in accordance to our hypothesis of the early aging. This difference is attenuated at older age where the bone pathology is

present even without hypertension. This is compatible with genetic influence, more evident at younger age with environment overriding later.

In our current data, we unveiled that augmentation index, a marker of arterial stiffness was significantly associated with height and it contributed to 20% of height differences. A study done by Reeve et al. showed that taller people have better central hemodynamics and reduced cardiovascular risks.[26] Ageing leads to various changes in the cardiovascular system including the augmentation in arterial stiffness and in pulse pressure, therefore high Aix and cPP are good indicators of aged vasculature and an increased arterial stiffness.[27] It is held that peripheral PP is a strong predictor of heart attack and stroke and it is often assumed that it reflects the central PP. In 2006, the CAFÉ study challenged that notion and showed that two BP lowering drugs had a similar impact on brachial BP but had a significantly different impact on reducing central aortic pulse pressure.[28] Those studies revealed the importance of cPP and its ability to better predict cardiovascular events (such as heart attacks and stroke). Moreover, in our study we observed a significant inverse association between arterial stiffness and BUA. Mangiafico et al. showed in a study where hypertensive women were excluded, that postmenopausal women with osteoporosis have an increased Aix and central pulse pressure in a small set of 182 osteoporotic and 160 controls, in spite of equal peripheral pressure.[29] Another small study carried by Sumino et al. showed the increased arterial stiffness in 25 osteoporotic postmenopausal women.[30] BUA measured by the quantitative ultrasound system reflects bone mineral density, thus in our study the inverse association between parameters of arterial stiffness and BUA in a large

population based cohort, and most importantly in both sexes, largely validates initial small studies in patients with demonstrated osteoporosis.

To our knowledge we are the first to show, in two population cohorts and one family cohort, the association between hypertension and height. In addition, the novelty of this study resides in the size of this cross sectional population cohort and the hypertensive and osteoporotic phenotypes that were gathered such as arterial stiffness and BMD in men and women. Our study presents a new opportunity to search for clinically relevant osteopenia in hypertensive men and women. The preventive intervention for progression of both arterial stiffness and fractures remains to be explored.

Conclusion

In conclusion considering the decreasing height and bone density associated with increased arterial stiffness and rate of fractures. Our study indicates that hypertension and osteoporosis are forms of accelerated ageing sharing at least in part pathophysiological processes. Our study also points out to the importance of evaluating bone health in hypertensive men.

Declaration of interest

We declare no conflict of interest.

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Ethics committee approval

Ethics approval was granted by the Faculty of Medicine of the University of Montreal and the Ethical research committee of the CHUM Research Center.

Authors and contributions

Dr. Pavel Hamet is the senior investigator of this study and for the Saguenay Lac Saint-Jean study.

Rana El Bikai was the principal executer of the study.

Dr. Ramzan Tahir was the statistical consultant.

Dr. Johanne Tremblay, Dr. Pierre Dumas, Dr. Ondřej Šeda, Dr. Lucie Šedová and Dr. Louis-Georges Ste-Marie were advisors for this study.

Dr. Claude Laberge, Dr. Bertha Maria-Knoppers and Dr. Philip Awadalla are the principal investigators for CARTaGENE.

Dr. Pavel Hamet was the medical director for CARTaGENE.

Dr. Michel Joffres was the principal investigator of the Canadian Heart Health Measure Survey.

Dr. Daniel Gaudet was the principal investigator of the Family cohort of the Saguenay Lac St-Jean.

Rana El-Bikai drafted the manuscript and all authors contributed to its revision and approved its final version.

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Tables and figures

Table 1: Description of categorical variables (CARTaGENE, N=18347)

	All	Normotensives	Hypertensives
		%(N)	
	100 (18347)	100 (12510)	100 (5837)
Age group			
40-54	58·3 (10694)	67·1 (8394)	39·4 (2300)
55-70	41·7 (7653)	32·9 (4116)	60·6 (3537)
Sex			
Men	48·5 (8898)	44·5 (5566)	57·1 (3332)
Women	51·5 (9449)	55·5 (6944)	42·9 (2505)
BMD			
Normal	81·7 (14991)	82·9 (10370)	79·2 (4621)
Low BMD	18·3 (3356)	17·1 (2140)	20·8 (1216)
Bone fractures			
No	86·9 (15945)	87·9 (11000)	84·7 (4945)
Yes	13·1 (2402)	12·1 (1510)	15·3 (892)
Antihypertensive medications			
No	79·9 (14654)	100 (12510)	36·7 (2144)
Yes	20·1 (3693)	0	63·3 (3693)
Osteopenia medications			
No	76·3 (14002)	77·3 (9672)	74·2 (4330)
Yes	23·7 (4345)	22·7 (2838)	25·8 (1507)
Osteoporosis medications			
No	96·5 (17710)	96·9 (12125)	95·7 (5585)
Yes	3·5 (637)	3·1 (385)	4·3 (252)

Figure 1: Height in normotensives and hypertensives in: a) Canadian Heart Health Study (CHHS) (N=23,129), b) Family cohort of the French Canadians (SLSJ) (N=849), c) CARTaGENE (CaG) (N=18,347).

N_N (number of normotensive participants); N_H (number of hypertensive participants); m_N (slope of height in normotensives); m_H (slope of height in hypertensives), the significance threshold for P value was set at 0·05.

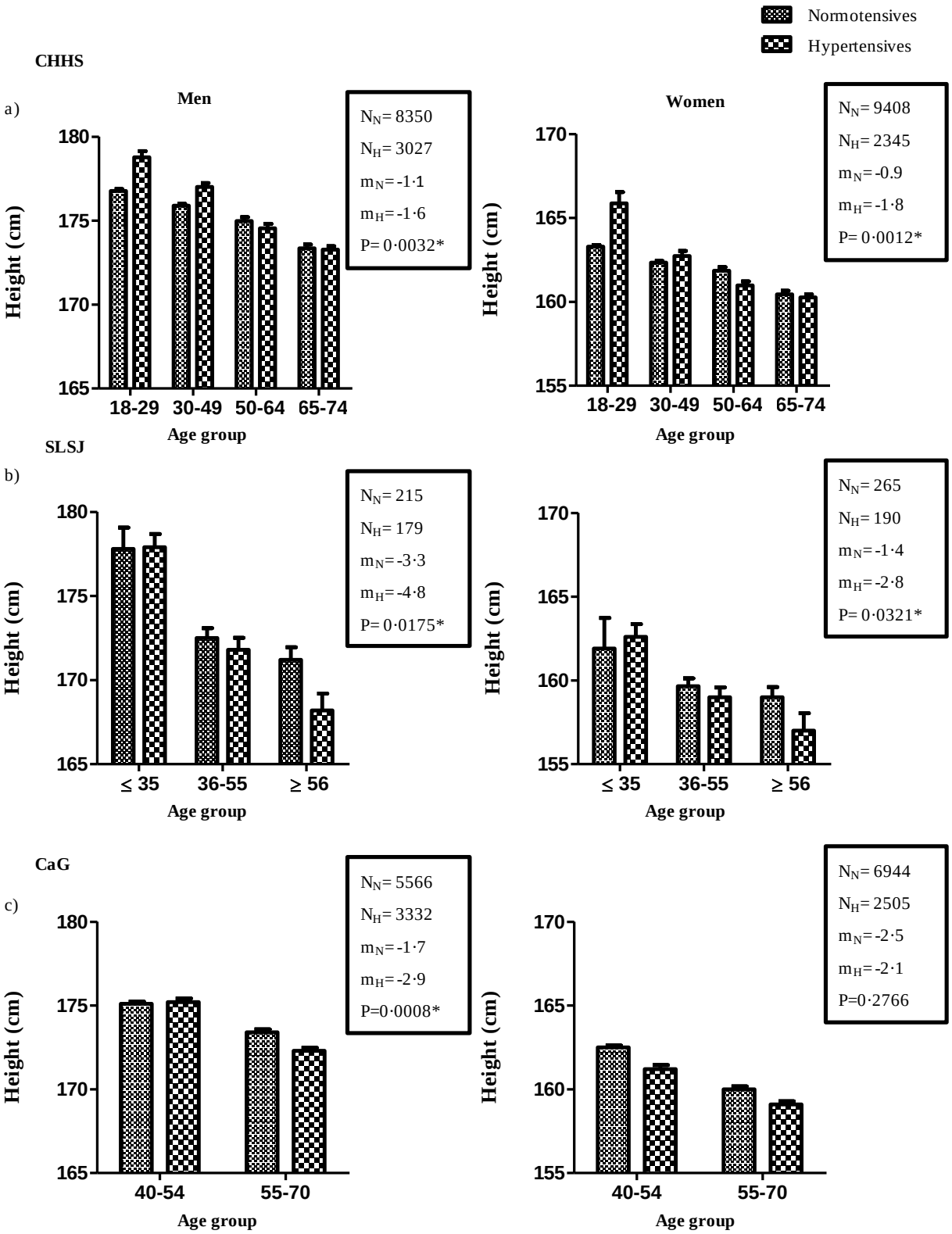
Normotensives
Hypertensives
CHHS
a)
Men
Women
Height (cm)
180
175
170
165
155
18-29
30-49
50-64
65-74
Age group
N_N= 8350
N_H= 3027
m_N= -1·1
m_H= -1·6
P= 0·0032*
N_N= 9408
N_H= 2345
m_N= -0.9
m_H= -1·8
P= 0·0012*
SLSJ
b)
≤ 35
36-55
≥ 56
N_N= 215
N_H= 179
m_N= -3·3
m_H= -4·8
P= 0·0175*
N_N= 265
N_H= 190
m_N= -1·4
m_H= -2·8
P= 0·0321*
CaG
c)
40-54
55-70
N_N= 5566
N_H= 3332
m_N= -1·7
m_H= -2·9
P=0·0008*
N_N= 6944
N_H= 2505
m_N= -2·5
m_H= -2·1
P=0·2766

Table 2: Mean of height in men and women according to blood pressure status in CARTaGENE.

	Normotensives	Hypertensives	
	Mean ± S.D		P value
	40-54		
Men	175·2±6·5	175·2±6·5	0·9448
Women	162·4±6·1	161·3±6·0	<0·0001
	55-70		
Men	173·4±6·5	172·4±6·5	<0·0001
Women	159·9±5·9	159·0±6·0	<0·0001

Table 3: Linear regression with height as dependent variable.

* Hypertension, peripheral diastolic blood pressure (pDBP), peripheral systolic blood pressure (pSBP), peripheral pulse pressure (pPP), central diastolic blood pressure (cDBP), central diastolic blood pressure (cSBP), central pulse pressure (cPP), augmentation index (Aix) coefficients' are adjusted for anti-hypertensive medications. And BUA coefficients' are adjusted for osteoporosis medications and anti-hypertensive medications

	Men						Women					
	Unadjusted data			Adjusted data			Unadjusted data			Adjusted data		
	40-54											
	β	SE (β)	P value	β*	SE (β)	P value	β	SE (β)	P value	β*	SE (β)	P value
Hypertension (1/0)	-0·01	0·21	0·9448	-0·21	0·27	0·4295	-1·03	0·21	<0·0001	-1·35	0·33	<0·0001
BUA	0·01	0·00	0·0181	0.01	0·01	0·0186	0·04	0·00	<0·0001	0·04	0·00	<0·0001
p DBP (mmHg)	-0·02	0·01	0·0075	-0·02	0·01	0·0064	-0·04	0·01	<0·0001	-0·04	0·01	<0·0001
p SBP (mmHg)	-0·01	0·01	0·1213	-0·01	0·01	0·1071	-0·03	0·00	<0·0001	-0·03	0·00	<0·0001
p PP (mmHg)	0·01	0·01	0·4327	0·01	0·01	0·4504	-0·03	0·01	0·0038	-0·03	0·01	0·0064
c DBP (mmHg)	-0·03	0·01	0·0025	-0·03	0·01	0·0022	-0·05	0·01	<0·0001	-0·05	0·01	<0·0001
c SBP (mmHg)	-0·05	0·01	<0·0001	-0·05	0·01	<0·0001	-0·05	0·01	<0·0001	-0·05	0·01	<0·0001
c PP (mmHg)	-0·09	0·01	<0·0001	-0·09	0·01	<0·0001	-0·07	0·01	<0·0001	-0·08	0·01	<0·0001
Aix (%)	-0·16	0·01	<0·0001	-0·16	0·01	<0·0001	-0·12	0·01	<0·0001	-0·12	0·01	<0·0001
	55-70											
	Unadjusted data			Adjusted data			Unadjusted data			Adjusted data		
	β	SE (β)	P value	β*	SE (β)	P value	β	SE (β)	P value	β*	SE (β)	P value
Hypertension (1/0)	-1·00	0·21	<0·0001	-1·13	0·29	0·0001	-0·95	0·19	<0·0001	-0·55	0·31	0·0736
BUA	0·01	0·01	0·0800	0·01	0·01	0·1095	0·04	0·01	<0·0001	0·04	0·01	<0·0001
p DBP (mmHg)	-0·00	0·01	0·7492	-0·00	0·01	0·6034	-0·02	0·01	0·0771	-0·01	0·01	0·1419
p SBP (mmHg)	-0·03	0·01	<0·0001	-0·03	0·01	<0·0001	-0·02	0·00	<0·0001	-0·02	0·00	0·0010
p PP (mmHg)	-0·05	0·01	<0·0001	-0·05	0·01	<0·0001	-0·03	0·01	0·0001	-0·02	0·01	0·0013
c DBP (mmHg)	-0·00	0·01	0·9875	-0·00	0·01	0·8225	-0·02	0·01	0·0953	-0·01	0·01	0·1607
c SBP (mmHg)	-0·04	0·01	<0·0001	-0·04	0·01	<0·0001	-0·04	0·01	<0·0001	-0·03	0·01	<0·0001
c PP (mmHg)	-0·09	0·01	<0·0001	-0·08	0·01	<0·0001	-0·06	0·01	<0·0001	-0·05	0·01	<0·0001
Aix (%)	-0·12	0·01	<0·0001	-0·12	0·01	<0·0001	-0·10	0·01	<0·0001	-0·10	0·01	<0·0001

Table 4: Logistic regression with fracture as dependent variable and BMD status as independent variable separated by age, sex and blood pressure status.

	Normotensives				Hypertensives			
	Men		Women		Men		Women	
	OR	CI	OR	CI	OR	CI	OR	CI
				40-54				
Low BMD	1·54	1·13-2·08	2·20	1·67-2·87	2·36	1·51-3·60	2·81	1·73-4·49
				55-70				
Low BMD	2·04	1·51-2·73	1·84	1·51-2·25	2·10	1·59-2·77	1·54	1·20-1·98

Table 5: Linear regression with BUA as dependant variable and cardiovascular parameters as independent variables.

* Hypertension, peripheral diastolic blood pressure (pDBP), peripheral systolic blood pressure (pSBP), peripheral pulse pressure (pPP), central diastolic blood pressure (cDBP), central diastolic blood pressure (cSBP), central pulse pressure (cPP), augmentation index (Aix) coefficients' are adjusted for anti-hypertensive medications.

	Unadjusted data			Adjusted data		
	β	**SE (β)**	**P value**	**β***	**SE (β)**	**P value**
Aix	-0·19	0·01	<0·0001	-0·17	0·01	<0·0001
cPP	-0·13	0·01	<0·0001	-0·11	0·01	<0·0001
PP	-0·05	0·01	<0·0001	-0·04	0·01	<0·0001

Table 6: Linear regression with BUA as dependant variable and cardiovascular parameters as independent variables separated by age and sex.

*Cardiovascular parameters coefficients' are adjusted for osteoporosis medications and anti-hypertensive medications

	Men						Women					
	Unadjusted data			Adjusted data			Unadjusted data			Adjusted data		
	β	SE (β)	P value	β*	SE (β)	P value	β	SE (β)	P value	β*	SE (β)	P value
						40-54						
Aix	-0·07	0·02	0·0008	-0·07	0·02	0·0010	-0·10	0·02	<0·0001	-0·10	0·02	<0·0001
cPP	-0·03	0·03	0·2147	-0·03	0·03	0·1805	-0·11	0·03	<0·0001	-0·10	0·02	<0·0001
PP	-0·00	0·02	0·9733	-0·00	0·02	0·9067	-0·08	0·02	0·0004	-0·08	0·02	0·0005
						55-70						
Aix	-0·06	0·02	0·0181	-0·06	0·02	0·0145	-0·05	0·03	0·0330	-0·06	0·02	0·0213
cPP	-0·06	0·02	0·0071	-0·05	0·02	0·0131	-0·10	0·02	<0·0001	-0·09	0·02	<0·0001
PP	-0·05	0·02	0·0276	-0·04	0·02	0·0431	0·09	0·02	<0·0001	-0·08	0·02	<0·0001

Supplement

A. Description of categorical variables by blood pressure status, sex and age (CARTaGENE, N=18,347).

	Normotensives		Hypertensives		Normotensives		Hypertensives	
	Men	Women	Men	Women	Men	Women	Men	Women
	40-54				55-70			
	% (N)							
	100 (3749)	100 (4628)	100 (1331)	100 (963)	100 (1802)	100 (2310)	100 (1996)	100 (1539)
BMD								
Normal	88·8 (3331)	88·2 (4082)	87·6 (1166)	85·8 (826)	83·0 (1496)	62·4 (1442)	83·0 (1657)	62·7 (965)
Low BMD	11·1 (418)	11·8 (546)	12·4 (165)	14·2 (137)	17·0(306)	37·6 (868)	17·0 (339)	37·3 (574)
Bone fractures								
No	90·4 (3390)	92·2 (4268)	89·5 (1191)	89·5 (862)	84·0(1513)	78·3 (1809)	83·5 (1667)	79·1 (1218)
Yes	9·6 (359)	7·8 (360)	10·5 (140)	10·5 (101)	16·0 (289)	21·7 (501)	16·5 (329)	20·8 (321)
Antihypertensive medications								
No	100 (3749)	100 (4628)	51·1 (680)	38·0 (366)	100(1802)	100 (2310)	32·7 (653)	28·7 (442)
Yes	0	0	48·9 (651)	62·0 (597)	0	0	67·3 (1343)	71·3(1097)
Osteopenia medications								
No	94·6 (3546)	75·5 (3492)	92·8 (1235)	73·1 (704)	86·4 (1556)	45·9 (1061)	87·6 (1748)	41·4 (637)
Yes	5·4 (203)	24·5 (1136)	7·2 (96)	26·9 (259)	13·6 (246)	54·1 (1249)	12·4 (248)	58·6 (902)
Osteoporosis medications								
No	99·7 (3739)	98·6 (4563)	99·5 (1323)	98·5 (949)	98·6 (1777)	87·7 (2025)	98·5 (1967)	86·9 (1338)
Yes	0·3 (10)	1·4 (65)	0·5 (8)	1·5 (14)	1·4 (25)	12·3 (285)	1·5 (29)	13·1 (201)

B. Description of continuous variables (CARTaGENE, N=18,347).

	All	Normotensives	Hypertensives
		Mean (SD)	
Age	54·2 (7·9)	52·5 (7·4)	57·6 (7·6)
Height (cm)	167·5 (9·2)	167·4 (9·1)	167·7 (9·3)
T-Score	0·2 (1·2)	0·2 (1·1)	0·1 (1·2)
pSBP (mmHg)	123·9 (15·9)	118·3 (11·2)	135·9 (17·0)
pDBP (mmHg)	73·8 (10·2)	71·2 (8·3)	79·3 (11·6)
pPP (mmHg)	50·2 (10·9)	47·2 (8·2)	56·6 (12·9)
cSBP (mmHg)	114·2 (15·0)	109·0 (10·8)	125·2 (16·5)
cDBP (mmHg)	74·9 (10·3)	72·3 (8·3)	80·5 (11·8)
cPP (mmHg)	39·2 (10·1)	36·7 (7·6)	44·8 (12·3)
Aix (%)	27·5 (10·8)	27·1 (10·9)	28·2 (10·7)
Weight (Kg)	77·5 (17·0)	74·7 (15·8)	83·3 (17·9)

C. Description of continuous variables by hypertension status, sex and age (CARTaGENE, N=18,347).

	Normotensives		Hypertensives		Normotensives		Hypertensives	
	Men	Women	Men	Women	Men	Women	Men	Women
	40-54				55-70			
	Mean (SD)							
Height (cm)	175·2 (6·5)	162·4 (6·1)	175·2 (6·5)	161·3 (6·1)	173·4 (6·5)	159·9 (5·9)	172·4 (6·5)	159·0 (6·0)
T-Score	0·4 (1·2)	0·3 (1·1)	0·3 (1·2)	0·2 (1·1)	0·2 (1·2)	-0·3 (1·1)	0·2 (1·2)	-0·2 (1·1)
pSBP (mmHg)	121·8 (9·2)	113·3 (11·1)	137·5 (15·0)	130·8 (17·5)	124·0 (9·6)	118·4 (11·5)	138·3 (16·8)	134·4 (17·9)
pDBP (mmHg)	73·3 (7·8)	70·1 (8·4)	83·8 (11·1)	82·0 (11·3)	72·0 (7·8)	69·3 (8·3)	77·6 (11·3)	75·9 (11·0)
pPP (mmHg)	48·5 (6·9)	43·2 (7·3)	53·7 (10·1)	48·9 (11·6)	52·1 (7·5)	49·1 (9·0)	60·8 (12·1)	58·4 (13·9)
cSBP (mmHg)	109·8 (9·5)	105·7 (11·1)	124·6 (14·9)	122·7 (16·7)	73·1 (7·8)	110·8 (11·2)	126·5 (16·6)	125·6 (17·3)
cDBP (mmHg)	74·4 (7·9)	71·3 (8·5)	85·1 (11·3)	83·3 (11·2)	113·3 (9·7)	70·4 (8·3)	78·7 (11·5)	77·0 (11·2)
cPP (mmHg)	35·4 (6·5)	34·4 (6·9)	39·6 (9·3)	39·4 (10·7)	40·2 (7·4)	40·4 (8·5)	47·7 (11·9)	48·7 (13·1)
Aix (%)	20·3 (9·6)	30·4 (10·0)	21·9 (10·0)	31·5 (10·1)	25·2 (9·4)	33·0 (9·4)	26·8 (9·9)	33·5 (9·2)
Weight (Kg)	83·6 (14·4)	68·4 (14·1)	91·3 (17·3)	79·4 (19·6)	81·5 (13·6)	67·9 (13·2)	87·0 (15·5)	74·1 (15·5)

Manuscript 2 (in preparation)

Title

Temporal aspects of development of hypertension and low bone volume in SHR and recombinant inbred strains of rats.

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Abstract

Hypertension is one of the major risk factors for cardiovascular diseases. The most widely used animal model that best displays high blood pressure and resembles to what is seen in human essential hypertension is the spontaneously hypertensive rat (SHR). In addition to their hypertensive phenotype this inbred strain shows many defects related to calcium handling and abnormal bone mineralisation. Our current project focuses on studying the common determinants related with the progression of hypertension and the changes in bone structural parameters associated to osteoporosis. Therefore, the SHR strain and the SHR-derived recombinant inbred strains (RIS), the HXB / BXH set, served as our genetic model to study these two phenotypes. Systolic, diastolic, as well as mean arterial pressure (MAP) were assessed by telemetry measurements at 3, 6, 9 and 12 months of age of 3 RIS and the SHR. The structural parameters of the proximal tibia bone were obtained from in vivo micro-CT scans in the same animals to assess the microarchitectural changes. Additionally we assessed the genetic determinants of bone parameters in RIS. Our data indicated the existence of strain differences in the temporal dynamics of bone parameters as well as in blood pressure. Single locus on chromosome X was associated to bone volume. In addition, we observed a significant correlation between bone volume and changes of blood pressure induced by L-NAME treatment. In conclusion, our phenotypic and genetic study opens new venues to study the underlying shared genetic determinants between low bone density and hypertension.

Introduction

Hypertension is the leading cause of cardiovascular mortality which accounts for 30% of total deaths in the world (1). Essential hypertension is a multifactorial trait that results from complex interplay between genetic determinants and environment. The spontaneously hypertensive rat (SHR) has become one of the most commonly used models for studying essential hypertension since its original derivation in 1960s (2). Apart from raised blood pressure, the SHR shows additional hemodynamic, humoral and metabolic disturbances similar to the human condition, including dysregulations of mineral metabolism and handling. McCarron et al. observed that SHR displayed low serum calcium and an increased calcium excretion in addition to increased parathyroid hormone levels compared to WKY rats (3). Other studies focusing on calcium metabolism and its relation to hypertension demonstrated the positive impact of high-calcium diet on lowering blood pressure in SHR (4). It was then proposed by MacGregor et al. that the increase in kidney stones in essential hypertension is due to the negative calcium balance that is caused by an increase in urinary calcium excretion (5). Calcium is an important metabolite and its imbalance has multiple implications in different pathologies such as hypertension and osteoporosis. These two diseases cause major health burdens on the aging society. Therefore, SHR seems to be the relevant model to assess both conditions as in addition to essential hypertension it has been reported to have impaired bone health. A study done by Izawa et al. suggested that spontaneously hypertensive rats develop osteoporotic bones at 26 weeks of age. They observed that SHR displayed a significantly lower bone volume compared to WKY rats suggesting the

development of osteoporosis in SHR (6). Significant reduction in trabecular bone volume and an increased osteoclastic activity in SHR compared to WKY has been also reported, emphasizing the low bone mass in this hypertensive animal model (7). A comprehensive tool for genetic analysis of complex traits based on SHR is the recombinant inbred strain (RIS) rat panel HXB/BXH (8). Two highly inbred strains were first crossed, the SHR and the normotensive Brown Norway (BN-*Lx*/Cub) (9) and the RIS were then produced by inbreeding brothers and sisters of the F2 generation for more than 40 generations thus fixing in each recombinant inbred strain a unique combination of the progenitors' genomes (6). One of the major advantages of using the RIS is their genetically identical background within the same strain, hence facilitating longitudinal studies. Extensive genetic studies on the RIS resulted in identifying numerous gene variants and quantitative trait loci (QTLs) that are involved in wide range of phenotypes such as hypertension, stress and insulin resistance (10-13). To date, more than 190 morphometric, biochemical and hemodynamic phenotypes along with transcriptomic profiles of major tissues have been assessed in the HXB/BXH panel (http://www.genenetwork.org/), yet no data are available for bone density parameters and there is only modest amount of longitudinal measurements for the available traits. We previously showed that hypertension is a form of accelerated aging assessed by an increased proliferation of aortic smooth muscle cells and an increased cellular turnover and apoptosis in kidneys, heart and the brains of SHR (14-16). Our current study aimed at following both female and male SHR rats and three recombinant inbred strains (HXB3, HXB13 and HXB17) selected for their distinct age of onset of hypertension and salt

retention for a period of one year. During the course of the study, we have assessed the changes of blood pressure, bone mineral density, mineral handling and insulin sensitivity on the defined genomic backgrounds of inbred strains.

Material and Methods

Animal model

We used one of the largest set of recombinant inbred strains (RIS) of rats, derived from the cross of SHR/OlaIpcv (represented by H) and BN-*Lx*/Cub rats (represented by B). Two sets of RIS were created; the HXB (representing a female SHR crossed with a male BN-*Lx*) and BXH (representing a female BN-*Lx* crossed with a male SHR). The RIS were created at the Institute of Biology and Medical Genetics of the First Faculty of Medicine, Charles University in Prague and Institute of Physiology of the Academy of Sciences of the Czech Republic. The inbred crossing (brother and sister mating) of the F2 generation was done for over than 40 generations producing 36 RIS displaying different combinations of the parental genotypes (8). The RIS in this study were selected based on our previous work, of their distinct age of onset of hypertension and salt retention (17); hence the aging component of the present work was performed using the male and female groups of SHR, HXB17, HXB13 and HXB3 strains. The phenotyping and all experiments were conducted at 3, 6, 9 and 12 months of age. Additional groups of rats were sacrificed at 3 months and 12 months of age (HXB17, HXB13 and HXB3) for bone histomorphometric analysis. All procedures were approved by the Institutional Animal Protection Committee of the Research Center of the University of Montreal (CRCHUM).

Blood pressure

Radiotelemetry (Data Sciences International, St-Paul, MN, USA) was used for blood pressure monitoring in all 32 strain*sex*age groups. The telemetry probe was inserted in the abdominal cavity with a catheter inserted in the femoral artery. Rats were left for ten days of recovery, after which continuous blood pressure reading started for three consecutive days, as described in detail previously (18). Distinct groups of animals were used for blood pressure assessment and bone mineral density and metabolic profiles.

Bone mineral density

The proximal tibias of all sex * age groups of SHR, HXB3, HXB13 and HXB17 strains were imaged by micro-CT (SkyScan 1176, Antwerp, Belgium) with a 1 mm Al filter and 65 kV, 385 μA were applied. The scan was performed with a standardised offset from the growth plate of the tibia. Metaphyseal volumes of interest for trabecular bone were selected. Trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp.) and structural model index (SMI) were analysed. For genetic analyses, an additional group of 3-month-old rats consisting of 7 male RIS (HXB26, HXB29, BXH2, BXH5, BXH9, BXH11, BXH13) and the BN-*Lx*/Cub parental strain had their tibias scanned and the relevant bone parameters calculated.

Metabolic Cages

To normalise the variation between the rats, the animals were kept for 36 hours fasting, with free access to water (4-6 rats per strain, per age). The rats were kept individually in metabolic cages for the whole duration, after which blood and urine samples were collected for sodium, calcium and potassium measurements.

Oral Glucose Tolerance Test (OGTT) and insulin measurement

OGTT was performed after 36 hours fasting. At the beginning of the procedure, intragastric glucose was administered to conscious rats (3 g/kg total body weight). Blood was withdrawn from the tail vein for glycemia determination by a glucometer (Abbott, Precision Xtra) at 0, 30, 60, 120 and 180 minutes after the bolus administration. Insulin concentration was measured by radioimmunoassay using a human insulin standard (Linco Research, St. Charles, MO).

Genetic analyses

We have performed linkage analyses and heritability estimation of BV/TV and Tb.N traits in the set of data from HXB3, HXB13, HXB17, HXB26, HXB29, BXH2, BXH5, BXH9, BXH11, BXH13 and the progenitor strains SHR and BN-*Lx*. We have performed the marker regression analysis, interval (QTL) mapping and the epistatic QTL mapping (Pair-scan) procedures using the online interface of WebQTL / GeneNetwork (http://www.genenetwork.org/) that contains the database of genotypes and phenotypes gathered in HXB/BXH recombinant inbred strains. In order to determine the genome-wide significance levels for linkage signals, 2000 permutations per trait were performed. Heritability was calculated according the following formula: $h^2=V_g/(V_g+V_e)$ where V_g represents the intra-strain variance (genetic variance) and V_e represents the inter-strain variance (environmental variance).

Results

Telemetry and bone morphology

The radio-telemetry results show distinct courses of blood pressure while aging between strains and sexes. The onset of hypertension was at an early stage of life in all

four rat strains and blood pressure continued to increase with age, particularly in females. In males, SHR started with significantly higher values of diastolic and systolic blood pressure (DBP and SBP respectively) and a higher mean arterial pressure (MAP) compared to the other strains and kept this distinction throughout the course of the study (***Figure 1a)***. On the other hand, in females we observed that all the strains had a comparable initial level of blood pressure and then increased distinctively depending on the strain, with SHR being the most hypertensive of all and HXB13 reaching relatively lowest values of blood pressure (***Figure 1b)***. In the second step, we analysed the strain- and time course-dependent changes in the bone micro-architectural parameters using the micro-CT. Tibias of 3 RIS and SHR were scanned and the trabecular bone volume and number along with the structural model index were measured. We observed that in males and females, HXB13 had the lowest BV/TV and Tb.N and had the highest rod like shape bone as indicated by the high values of SMI. In males, we observed that HXB17 started at 3 months of age with the highest bone volume and it decreased with time to have the lowest bone parameters after HXB13. At the same time, HXB17 had the highest blood pressure after the SHR (***Figure 1a)***. In females, the same pattern as in HXB 17 was observed for the HXB3, the latter displayed the highest blood pressure among the female RIS and in parallel with the onset of hypertension it developed a low BV/TV and Tb.N along with an increase in its rod like shape bone (***Figure 2b)***. Micro-CT scanning was followed by histomorphometric analysis for confirmation (data not shown). The analysis showed the same pattern of bone volume and Tb.N decrease and an increase in trabecular separation while aging. In parallel, we investigated the excretion of two

important metabolites (sodium and calcium) that are directly related to both pathologies, hypertension and bone loss. We observed strain-specific courses of the fractional excretion of sodium as evident from (***Figure 3)*** with a particularly prominent decrease in HXB17 males and all female rats indicating an increase of sodium retention while aging This decrease in sodium excretion was paralleled with an increase in calcium excretion in both genders as shown in (***Figure 4)***. The calcium excretion in HXB17 was significantly highest across all time points in both sexes.

Insulin resistance and glucose levels

In males, the glucose tolerance deteriorated along age, especially in SHR (***Figure 5a)***. Fasting insulin concentration diminished dramatically at 12 month in SHR, while the highest concentration for the other three strains was reached at this age (***Figure 6a)***. In females, the pattern was slightly different; insulin resistance was observed in SHR at 3 and 12 month of age, while the other strains seemed to develop this pathology at 12 month of age (***Figure 5a).*** Insulin concentration increased with age remarkably in SHR to reach its highest levels at 12 month of age. HXB13 had the highest levels of insulin at 3 month of age and it diminished with age. We also observed that HXB3 reached the highest concentration of insulin at 6 and 9 month of age. HXB17 had an unchanged level of insulin at all 4 time points of its age ***(Figure 6b)***.

Genetic analysis

In an extended set of recombinant inbred strains, we have assessed the genomic determinants of bone density. The strain distribution pattern displays a strong gradient and a continuous distribution for both BV/TV and Tb.N traits. Heritability estimation

showed that both traits have a substantial genetic contribution, the h^2 for BV/TV and Tb.N being 53.9% and 60.7% respectively (***Supplement Figure 1***). Our linkage analysis showed a significant quantitative trait locus (QTL) on X chromosome for the trabecular bone volume trait. Spearman correlation analysis of BV/TV with the published phenotypes for HXB/BXH set (192 phenotypes) showed that the only blood pressure phenotype positively which correlated with BV/TV was the delta N^G-nitro-L-arginine-methyl ester (L-NAME) effect (mmHg), in 12-week-old males as shown in (***Table 1a).*** No blood pressure phenotypes were significantly correlated with Tb.N (***Table 1b)***. We have detected significant linkage for BV/TV to a single locus on X chromosome. No significant hits in pair-scan were observed (***Table 2)***. Tb.N had several suggestive linkage signals on X chromosome for marker regression and interval mapping analyses. Epistatic interaction scan revealed a suggestive interaction between loci on chromosomes 1 and X (***Table 3)***. Since HXB17 displayed rather distinct phenotypic profile from the rest of the RIS, particularly in mineral handling, we have utilized the available comprehensive genotypig data of > 20,000 SNPs to identify genomic regions distinguishing HXB17 from HXB3 and HXB13. The span of multiple regions is summarized in Supplementary Table X.

Discussion

Blood pressure and bone

Different human and animal studies investigated the co-existence of hypertension and bone loss (6, 19-21). To our knowledge, we are the first to investigate the relation between high blood pressure and low bone mineral density in a longitudinal study (12 month follow up) on SHR and the recombinant inbred strains of male and female rats.

Wang et al. carried a study on 25-week-old male SHR and their normotensive controls, the Wistar-Kyoto (WKY) rats. The histomorphometric analysis showed that the SHR had a significantly lower bone parameters compared to WKY (7). Another study characterized the trabecular bone turnover in male and female SHR, WKY and Sprague Dawley rats (SD) at 8 weeks of age and at 24 weeks. These results demonstrated a severe resorbtive activity in both sexes of SHRs compared with their controls (22). Other studies carried on human and animal models, reported a calcium leak in hypertensives, and an imbalance in calcium handling (3, 4, 23). Our results arethus corroborating the previous observations in our multi-timepoint setup, as we observed a development of low trabecular volume and number and an increase in the gaps between the trabeculae while aging. Those changes in bone micro-architecture and the increase in calcium excretion were observed to go in parallel with the early onset of hypertension in the different strains and in both sexes.

Blood pressure and insulin

Hypertension is often accompanied with metabolic syndrome features such as insulin resistance. Insulin resistance and hyperinsulinemia were seen in SHR by different research groups (24-26). It is observed that cells become resistant to glucose uptake in the adipose tissues in SHR and they have a defect in lipid metabolism (26, 27). Insulin resistance, glucose uptake and fatty acid metabolism impairment were mapped to single locus on the rat chromosome 4 in SHR. This led to the identification of the mutated *Cd36*/*Fat* gene which underlies insulin resistance (28, 29). Transgenic rescue using wild-type *Cd36*/*Fat* in SHR was observed to ameliorate insulin resistance and

lower the levels of fatty acids in the serum (30). Observations of our study are in concordance with what was reported by others, since we observed an increase in insulin concentration, and a reduction in glucose uptake following OGTT indicating the presence of insulin resistance in SHR and RIS, particularly in HXB17.

SDP and QTL

The recombinant inbred strains of rats were created to study phenotypic and genetic pathologies. Many genetic analyses were achieved to identify QTLs and candidate genes that are responsible for the phenotypes observed in SHR, BN-*Lx* and RIS such as hypertension, insulin resistance, electrolyte excretion and other traits (1, 7, 13). Bone–density related traits have not yet been reported in the HXB/BXH set. In this study we showed that SHR as well as three RIS developed a very low bone volume and trabecular number and an increased structural model index in parallel with the onset of hypertension while aging. The result of our linkage study indicated the presence of a significant QTL for BV/TV trait on the X chromosome. Since the linked locus does not fall into either of the pseudoautosomal regions of the X chromosome, it can be deduced that the linkage signal represents the effect of X chromosome origin in the respective strains. In this respect it should also be noted that along with the X chromosome, the maternal mitochondrial DNA (mtDNA) is inherited in the sex-specific manner (i.e. SHR mtDNA in all HXB strains and BN-Lx mtDNA in all BXH strains). Pravenec et al. have previously demonstrated by derivation of SHR/OlaIpcv-mtBN/Crl conplastic strain that mtDNA variants can substantially influence complex traits including insulin resistance and blood pressure (31, 32).It follows that any systematic contrast between HXB and

BXH can be thus influenced by mtDNA (or Y chromosome (33) variants. In addition, we observed a significant interaction between chromosome 1 and X for the Tb.N trait. The chromosome 1 locus has been reported previously to be significantly linked to several parameters of bone structure and strength in a segregating F2 population of Copenhagen 2331 x Dark Agouti rats (34). X chromosome locus overlaps with a single previous report showing female-specific, genome-wide significant linkage of tibial area measured by two-dimensional dual-energy X-ray absorptiometry (DXA) in reciprocal F2 crosses between diabetic GK and normo-glycemic F344 rat strains (35) or for any other traits. Our results show a significant correlation between BV/TV and the delta of L-NAME effect on blood pressure that was observed in 12 weeks of age rats by Kuneš et al. (36). L-Name is known to block the nitric oxide synthase (NOS) thus increasing blood pressure. Nitric oxide (NO) exerts its beneficial effect on the endothelial cells thus inducing vasodilation, In addition, NO modulates the autocrine and paracrine activity on bone metabolism as it reduces osteoclasts activity and hence reduces porosity in bones (37, 38). The use of L-NAME blocks the activity of NOS thus increasing blood pressure and bone resorbtion. Tsukahara et al. explored the effect of L-NAME on bone and observed that bone mass was reduced following L-name treatment (39). The positive correlation that was observed in our study between bone volume and the delta change in blood pressure following L-NAME use, imply a possible pathophysiological link that should be pursued in further studies. Nitric oxide plays an important and protective role on the vasculature and on bone formation (40-42). Its reduced availability in cases of hypertension could explain the vasoconstriction that is very common in hypertensive

subjects and animal models and also explains reduced bone volume that is observed in SHR and RIS, particularly in HXB17.

Conclusion

Finally, the early and progressive onset of hypertension is accompanied by a decrease of bone volume, trabecular number and an increase in trabecular separation in animal models of hypertension (SHR and HXB/BXH set of RI rats). In addition, our study points out the presence of a significant correlation between bone volume and delta change in blood pressure following L-NAME treatment, suggesting the presence of shared pathophysiological mechanisms between hypertension and low bone volume. We have identified the HXB17 strain to possess a particular profile including high calcium excretion and temporal development of insulin resistance.

Limitations

The present study represents some limitations concerning the number of strains used in the genetic analyses, full set of RIS would likely enhance the robustness and probability of uncovering more variants.

Acknowledgement

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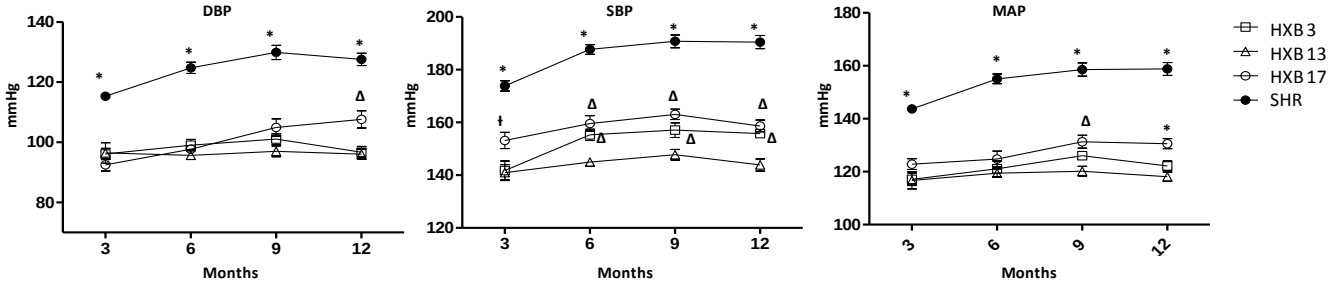
Figures and tables

Figure 1: Distribution of blood pressure (Diastolic blood pressure (DBP), Systolic blood pressure (SBP) and Mean Arterial Pressure (MAP)) in SHR (black circle), HXB 3 (White square), HXB 13 (white triangle) and HXB 17 (white circle) while aging in male (a) and female (b) rats. Significance levels for blood pressure by Two-way ANOVA, with the strain and age comparison using the Bonferroni post-test, significance is shown as follows: ł ($p<0.05$); Δ ($p<0.01$);* ($p<0.001$).

(a)



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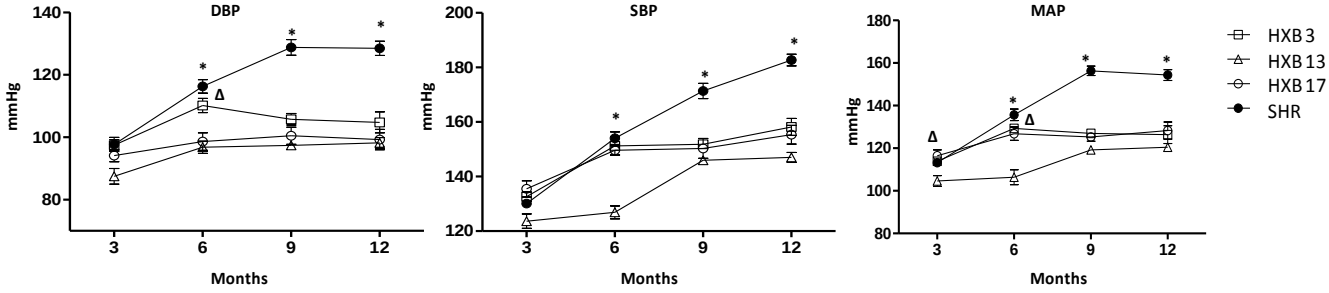
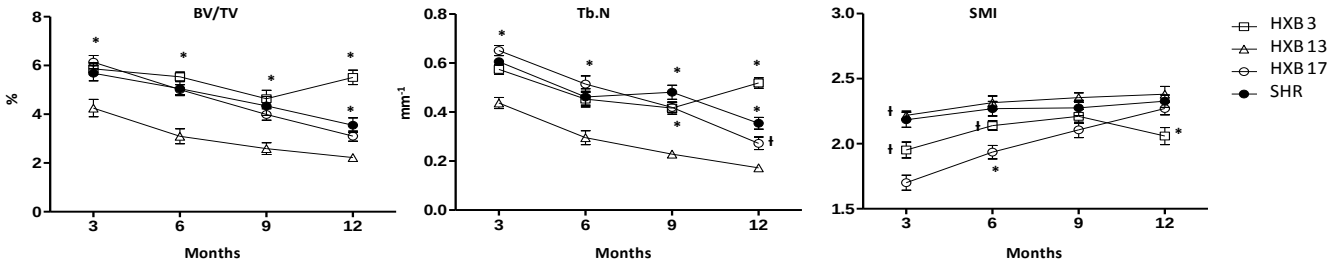


Figure 2: Distribution of bone parameters (Trabecular bone volume (BV/TV), Trabecular number (Tb.N) and Structural Model Index (SMI)) along with age in SHR (black circle), HXB3 (White square), HXB13 (white triangle) and HXB17 (whit circle) while aging in males (a) and females (b). Significance levels for bone parameters by Two-way ANOVA, with the strain and age comparison using the Bonferroni post-test, significance is shown as follows: ł ($p<0.05$); Δ ($p<0.01$);* ($p<0.001$).

(a)



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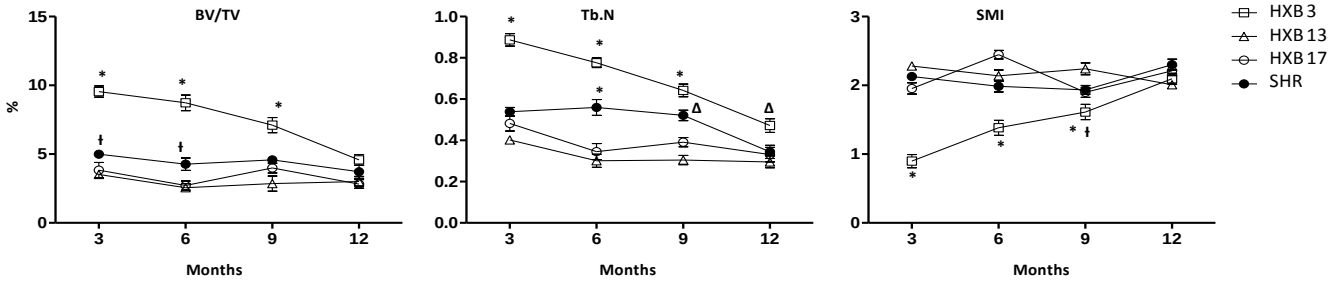
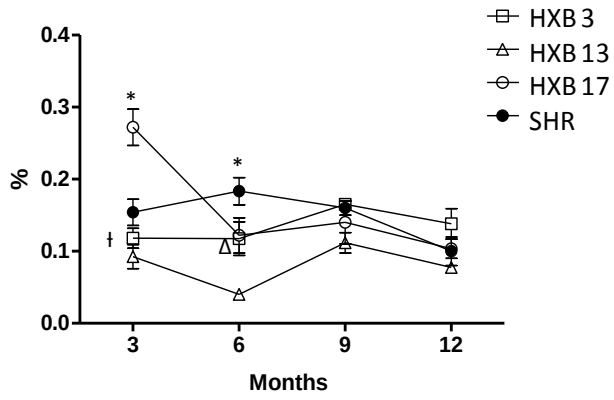


Figure 3: Fractional excretion of sodium in SHR, HXB3, HXB13 and HXB17 at 3, 6, 9 and 12 months of age in males (a) and females (b). Significance levels were assessed by Two-way ANOVA, with the strain and age comparison using the Bonferroni post-test, significance is set at P<0.05. ł (P<0.05); Δ (P<0.01);* (P<0.001).

(a)



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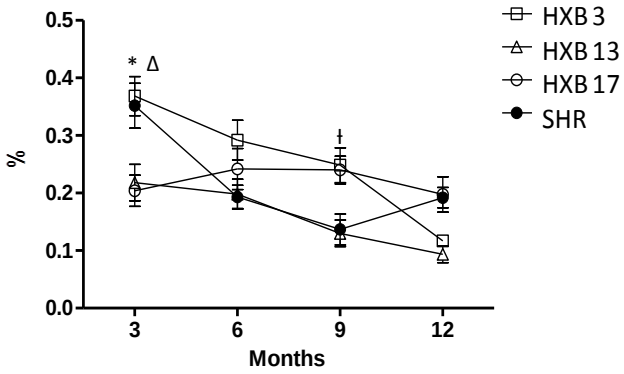
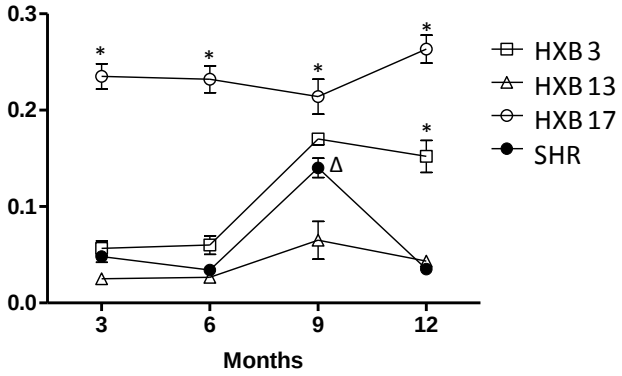


Figure 4: Calcium excretion (Calcium over creatinine ratio) in SHR, HXB3, HXB13 and HXB17 at 3, 6, 9 and 12 month of age in males (a) and females (b). Significance levels were assessed by Two-way ANOVA, with the strain and age comparison using the Bonferroni post-test, significance is set at P<0.05. ł (P<0.05); Δ (P<0.01);* (P<0.001).

(a)



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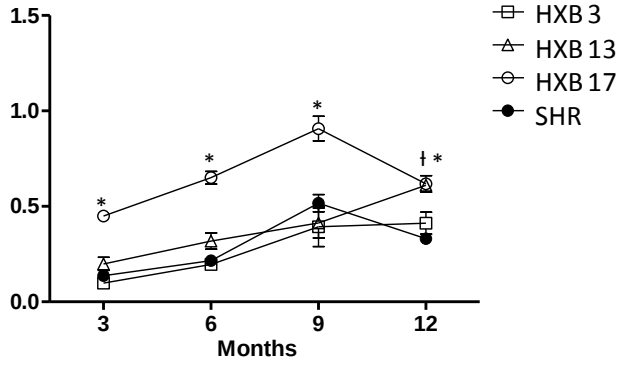
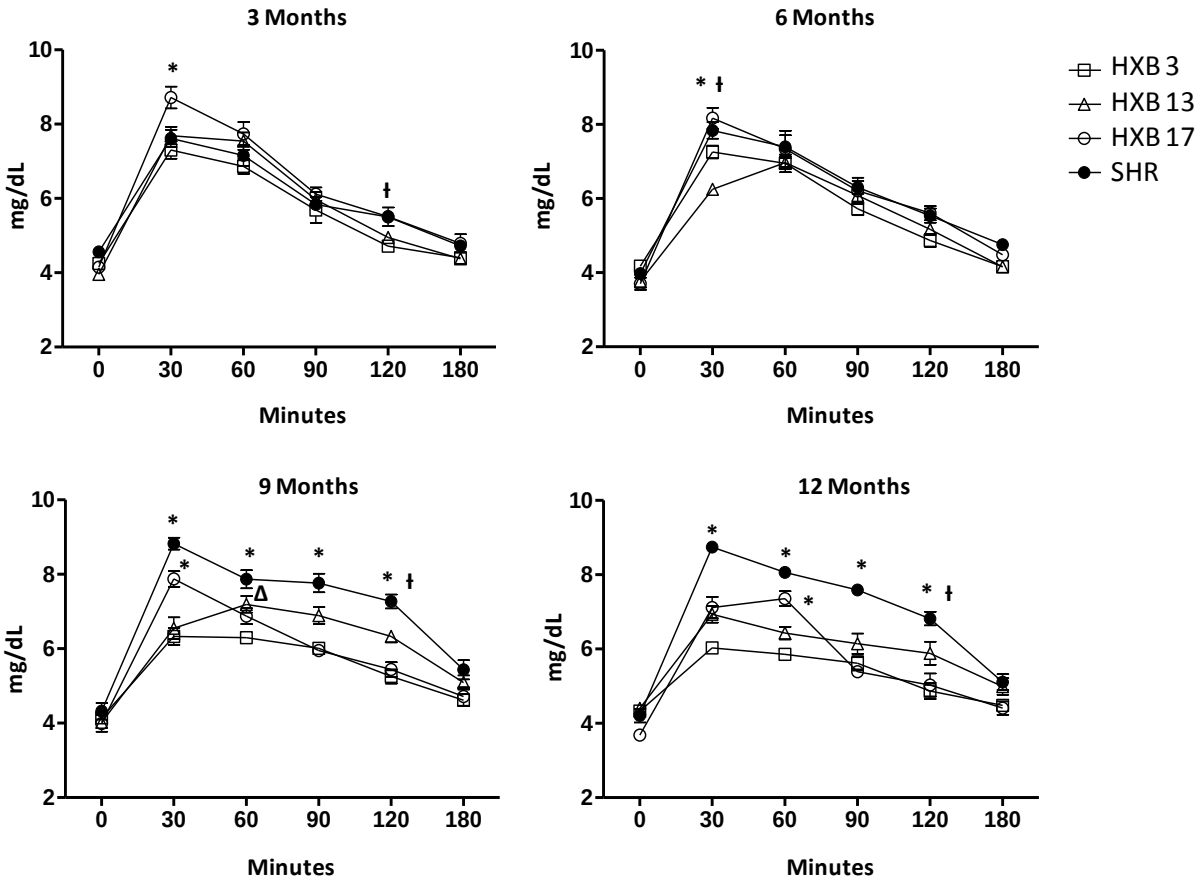


Figure 5: Oral Glucose Tolerance Test, represented by the glycaemia curve in SHR (black circle), HXB3 (white square), HXB13 (white triangle) and HXB17 (white circle) at 3, 6, 9 and 12 month of age in males (a) and females (b). Significance levels for glycaemia by two-way ANOVA, with the strain and age comparison using the Bonferroni post-test, significance is shown as follows: ł ($p<0.05$); Δ ($p<0.01$);* ($p<0.001$).

(a)



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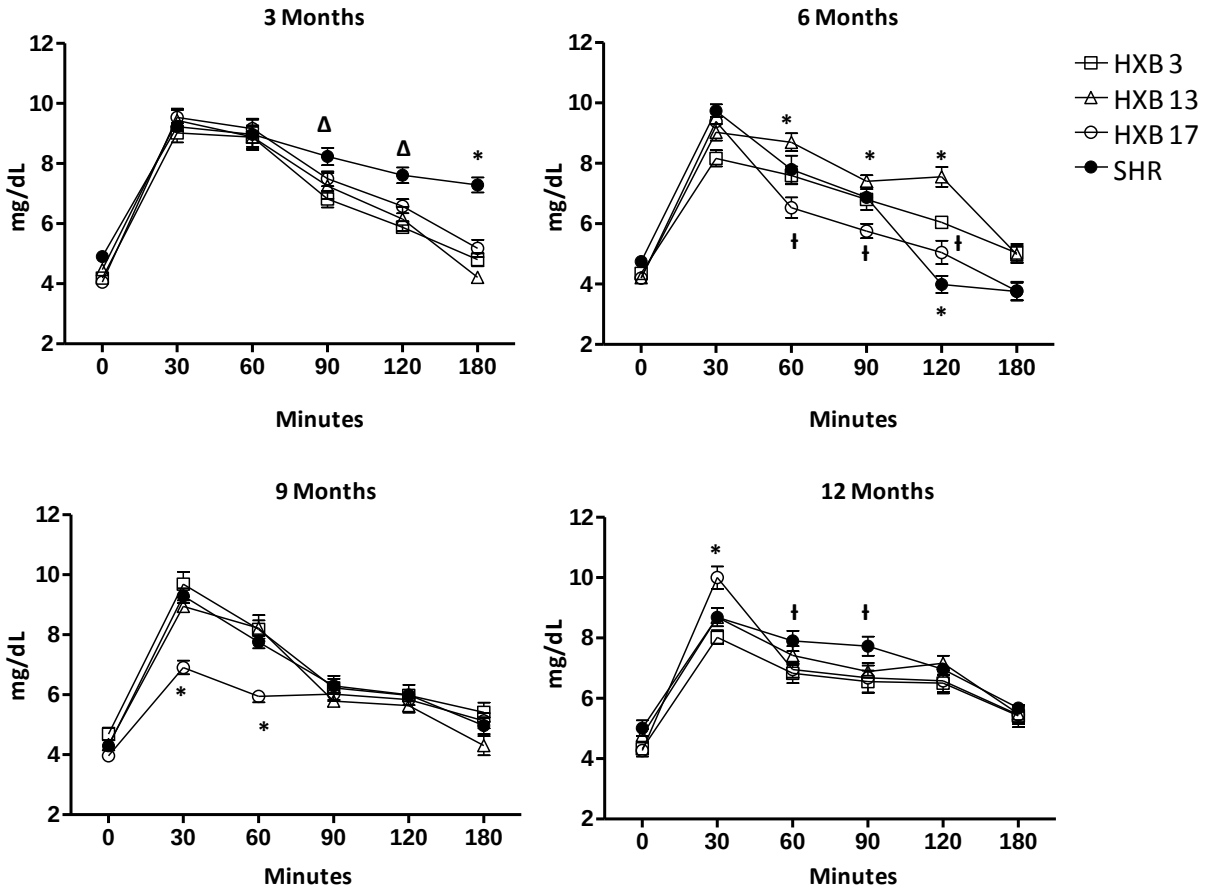
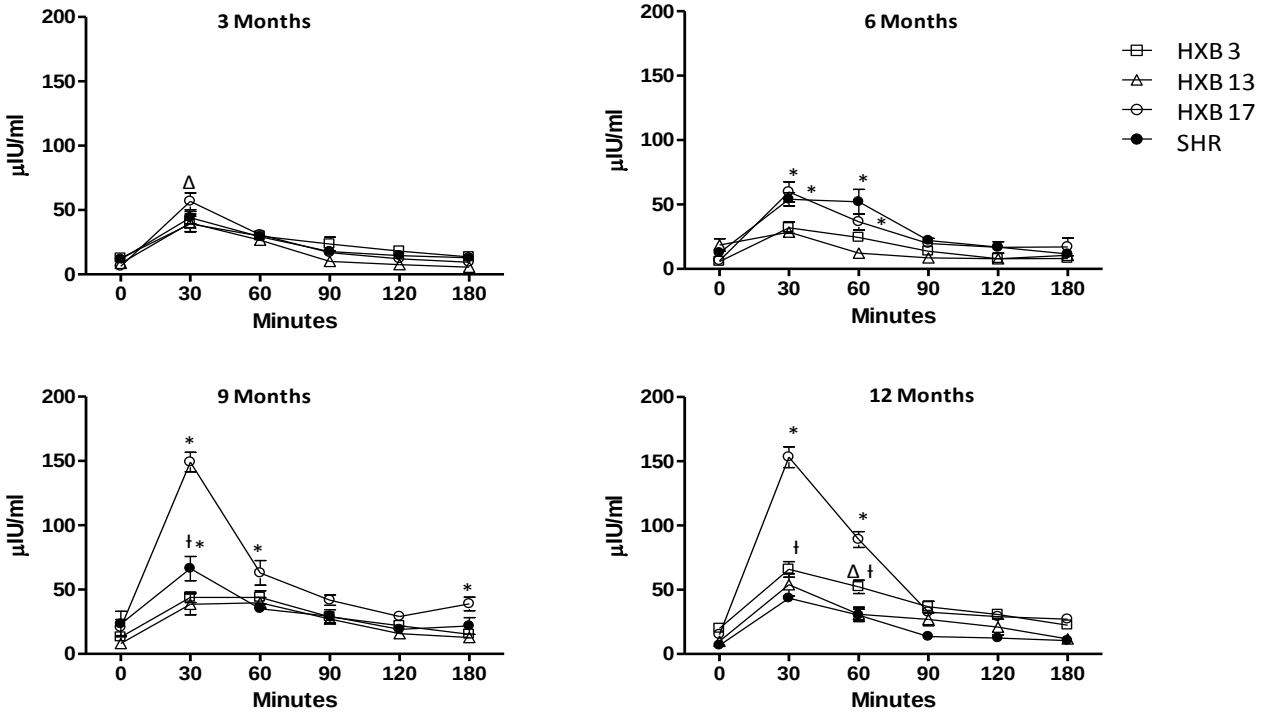

3 Months
6 Months
9 Months
12 Months
mg/dL
Minutes
0
30
60
90
120
180
2
4
6
8
10
12
HXB 3
HXB 13
HXB 17
SHR

Figure 6: Fasting insulin concentrations in HXB3, HXB13, HXB17 and SHR in males **(a)** and females **(b)**. Significance levels for insulin concentration were set by one-way ANOVA, with the strain comparison using the Bonferroni post-test, significance is shown as follows: : ł ($p<0.05$); Δ ($p<0.01$);* ($p<0.001$).

(a)



(b)

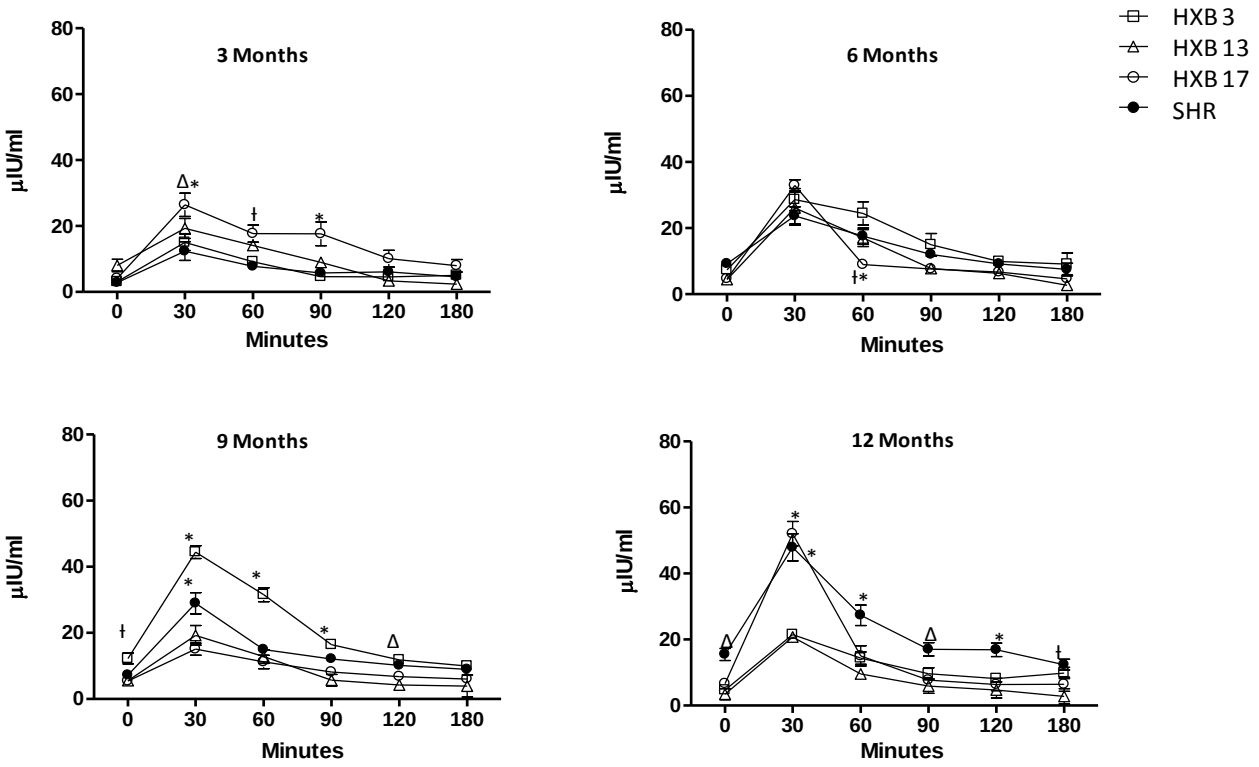

HXB 3
HXB 13
HXB 17
SHR
3 Months
6 Months
9 Months
12 Months
µIU/ml
Minutes
0
20
40
60
80
0
30
60
90
120
180

Table 1: Spearman correlation (Rho) of **(a)** BV/TV and **(b)** Tb.N with published phenotypes of HXB/BXH set.* unpublished results as available at HXB/BXH Published Phenotypes Database (www.genenetwork.org).

(a)

Phenotype	Rho	P value	N	Ref.
Glucose concentrations, 8 weeks old, fed a normal lab chow - male	-0.7818	0.00547	10	(10)
Body weight (g), males, 10 wk males	0.7182	0.01057	11	(Pravenec, Kazdova et al., unpublished results*)
Liver weight (g), 10 wk males	0.7182	0.01057	11	(Pravenec, Kazdova et al., unpublished results*)
Serum corticosterone levels after 80 min of immobilization stress in 10 weeks old males	-0.7857	0.01776	8	(Pravenec, Mormede, unpublished results*)
Body weight of mothers (g)	0.7619	0.02524	8	(43)
Fetus number in right horn of the uterus	0.7619	0.02524	8	(43)
Liver glutathione (µM/mg protein), 10 wk males	0.7167	0.02736	9	(Pravenec, Kazdova et al., unpublished results*)
Basal lipolysis glycerol levels (mmol/g tissue/2h), 10 wk males	-0.7000	0.03363	9	(Pravenec, Kazdova et al., unpublished results*)
Delta L-NAME effects (mm Hg), 12 wk males	**0.6182**	**0.04112**	**11**	**(36)**
Serum corticosterone levels after 40 min of immobilization stress in 10 weeks old males	-0.7143	0.04515	8	(Pravenec, Mormede, unpublished results*)

(b)

Phenotype	Rho	P value	N	Ref
Liver glutathione (µM/mg protein), 10 wk males	0.7500	0.01716	9	(Pravenec, Kazdova et al., unpublished results*)
Glucose concentrations, 8 weeks old, fed a normal lab chow - male	-0.6758	0.02979	10	(10)
Liver weight (g), 10 wk males	0.6364	0.03341	11	(Pravenec, Kazdova et al., unpublished results*)
Body weight (g), males, 10 wk males	0.6273	0.03713	11	(Pravenec, Kazdova et al., unpublished results*)

Table 2: Loci associated with BV/TV trait in the HXB/BXH set, after single marker regression analyses**.** LRS (likelihood ratio statistic). Highly significant association threshold (17.99) was computed using 2000 permutations.

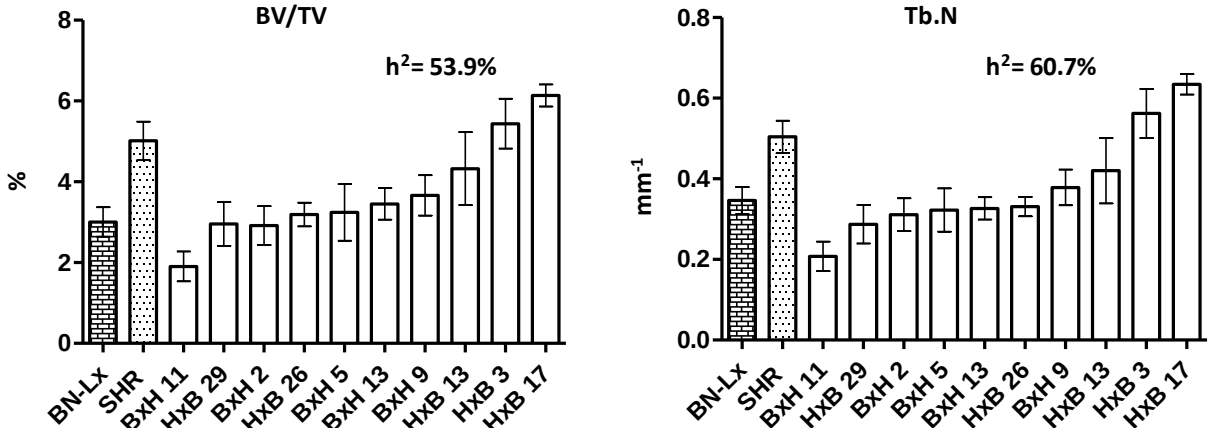
LRS	Chr.	Mb	Locus
17.986	X	20.000000	*DXUcsf2*
17.986	X	28.062012	*Mycs*
17.986	X	50.000000	*DXCebr7s16*

Table 3: Pair-Scan representing epistatic interaction for Tb.N trait. Chr.: chromosome; Mb: position on chromosome (LRS Full denotes the likelihood ratio statistic for the full model, the LRS Interact stands for the likelihood ratio statistic for the interaction.

Position		Flanking Markers			
Chr.	**Mb**	**Proximal**	**Distal**	**LRS Full**	**LRS Interact**
1	241.0-248.4	*D1Cebrp29s6*	*D1Arb25*	36.951	25.189
X	50-62.9	*DXCebr7s16*	*DXMit5*		

Supplement

Figure 1: Strain distribution pattern of BV/TV and Tb.N in 10 male RIS and the two parental (BN-*Lx* and SHR) strains at 3 month of age. Data are shown as mean ± SEM. h^2 denotes the heritability of the respective traits.



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Discussion

A. Body height and hypertension

The diagnosis of hypertension in children is challenged by debate as whether to consider age and weight in the reference range of normative values or to consider height alone. Most studies concluded that height and age are good indicators of growth and easily measured; it was therefore important to consider height in youth for the diagnosis of high blood pressure. It was proposed by Gillum et al. that in children between 6 and 9 years, age and height together should be taken into account when determining hypertension. In this study it was proposed that children in the top percentile of height demonstrated an elevated blood pressure in comparison with the other children of the same age (158). On the other hand in different small studies such as the population based study done by Reeve et al. with an average age of subjects of 67.5 years, showed that taller subjects are prone to have a lower blood pressure compared to short individuals. Augmentation index was in reverse correlation with height, in addition it was observed that taller participants had less prevalence of hypertension and use of antihypertensive drugs suggesting the beneficial role of height in estimating cardiovascular risks (159). In a study done on patients with end stage renal disease augmentation index was found to negatively correlate with body height, and it was found to be positively correlated with left ventricular hypertrophy. It was also indicated that lower body height and growth retardation are risk factors for emerging cardiovascular complications in end stage renal disease patients (160). When central blood pressure was measured and indicators of arterial stiffness were calculated, the

augmentation index was associated with a longer left ventricular ejection time and was inversely correlated with body height. Indicating that shorter subjects (men with a mean age of 46 years) were more prone to have an increased arterial stiffness (161). A genome wide analysis was carried by Andreassen et al. to assess the overlap between genes of systolic blood pressure and other disease. It was shown that SBP had small shared polygenetic effects with height (14). In summary, young children with higher blood pressure seem to be taller than their normotensives counterparts. In addition, older hypertensive subjects with an increased augmentation index were shown to be shorter than the ones with normal blood pressure. These preliminary suggestions of the link between hypertension, arterial stiffness and height were tested and ascertained by the results of our study.

B. Osteoporosis and height

Aging is accompanied by physical and pathophysiological changes, such as the development of osteoporosis, hypertension and other aging related diseases, in addition to a decrease in body height. Different studies tried to explain the reason behind the shorter stature acquired while aging, two facts underlying height shortening are explored: loss of bone mineral density and/or vertebral fractures. Hanson et al. and Kleerekoper et al. reported significant shortening in height along with a reduction in BMD, in longitudinal studies in postmenopausal women (162, 163). Xu et al. carried out a study on 231 men and women aged above 65 years on which they performed DXA for bone mineral density and vertebral fracture assessment. Their study showed that subjects with vertebral fractures had a higher height loss compared to the ones without

fractures and that was seen to be true in both men and women. Height was significantly associated with vertebral fracture and the magnitude of the association was higher in participants with more than one fracture (164). In a cross sectional study done on subjects from the Malaysian Aging Men study, 840 subjects were recruited with a mean of age of 47.3 years. Bone density measured on the calcaneus bone using the quantitative ultrasound system, the speed of ultrasound (SOS) was used in their analysis. It was shown that height was only negatively correlated with SOS in middle aged and older men but not in younger men indicating the decrease in bone density and height with age in men (165). Another study showed the correlation of higher height loss with the increase in the number of vertebral fractures. Patients could lose up to 6 cm from their original height if they have five or more vertebral fractures (166). The development of spinal osteoporosis is accompanied by a loss of height and this phenomenon is mostly seen in elderly (167, 168). All these small studies concord with what we have shown in a large population cohort, CARTaGENE, where height was significantly associated with BUA.

C. Hypertension, central blood pressure, low bone mineral density and fractures

Different studies investigated the relation between hypertension, low bone mineral density and fractures, but non explored hypertension, central blood pressure, low BMD and fractures in the same study, in a big population based cohort and in both sexes.

1. Hypertension

At the beginning, hypertension research and its relation to bone mineral density started by considering disturbance in sodium and calcium metabolism, observed in SHRs and in hypertensive patients (5, 7, 150, 155). Many studies showed the inter-relation between hypertension and other cardiovascular diseases with BMD. Recently a meta-analysis of 10 prospective cohort studies done by Qu et al. showed an association between low BMD and all causes of mortality and cardiovascular mortality. This implies the significant deleterious impact of the concomitant presence of cardiovascular diseases and low BMD (169). A review performed by Ghosh et al. on anti-hypertensive drugs, bone mineral density and fractures, explore the link between these pathologies. Since all anti-hypertensive drugs act positively or negatively bone density and fractures through different pathways and mechanisms, based on those observational evidences they seconded the existence of a link between hypertension and bone health (170). A longitudinal study carried on 1032 men and 1701 women investigated the relation between hypertension, fractures and BMD. Their analysis showed hypertension as a risk factor for fractures, independently from BMD in women. But in this study they did not reach statistical significance in the association between hypertension and fractures in men (171). Study of postmenopausal Turkish women, described the significant negative association between systolic blood pressure and BMD at the femur site (172). Sennerby et al. analysed association between cardiovascular diseases and hip fractures in the Swedish Twins Registry which included 15 968 twins. It was found that the highest hip fractures were found after diagnoses with heart failure or stroke. The increased number

of hip fractures in dizygotic twins after diagnosis of cardiovascular disease (CVD), implies the contribution of shared genetic factors between hip fractures and CVD (173). Building evidences are in agreement with the results of our study, where we observe a significantly higher rate of low BMD and fractures in hypertensive participants (men and women) in comparison to normotensives.

2. Arterial stiffness

Different physical measurements are important to the follow up of hypertensive patients and to monitor the efficacy of their treatment. In addition to peripheral blood pressure measurement, central blood pressure is seen to reflect more on the vascular health. Applanation tonometry is an easily accessible and non-invasive technique determining the shape of the aortic waveform from the radial artery and peripheral blood pressure to derive central arterial pulse wave, central blood pressure and other related parameters. A study done by Siebenhofer et al. showed that the use of this technique and its reproducibility in healthy adults is excellent. On the other hand, the variability observed between peripheral and central blood pressure was seen to be important suggesting the better role of central pressure to predict cardiovascular diseases (174). This observation was then ascertained by a clinical trial that was specifically designed for this purpose, the Conduit Artery Function Evaluation (CAFE) study. The latter is a study that recruited patients from the Anglo-Scandinavian Cardiac Outcome (ASCOT) study and patients were divided in two groups and received two different arms of combination treatments (amlodipine ± perindopril-based or athenolo ± thiazide based). Blood pressure monitoring in those patients, showed similar brachial

blood pressure but significantly different central blood pressure following their treatments which indicates the importance of central hemodynamic monitoring in hypertensive subjects (175). Considering the meaningful above mentioned evidences on central blood pressure surveillance, different studies investigated the possible relation between arterial stiffness and bone mineral density. Mangiafico et al. searched for the link between augmentation index (Aix), T-score (of the femoral neck and the lumbar spine), osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) in postmenopausal women with no cardiovascular diseases. It was shown that Aix was significantly inversely correlated with T-score but it did not correlate with OPG and RANKL (176). OPG and RANKL are known to be implicated in the bone remodelling; additional evidences showed that they are involved in cardiovascular remodelling and arterial calcifications (177-179). The lack of correlation between Aix, OPG and RANKL in their study implies a direct link between arterial stiffness and bone mineral regardless of arterial calcification. In another study carried by Masugata et al. on 52 hypertensive patients (27 men and 25 women) with a mean of age of 71, it was shown that arterial stiffness was significantly inversely associated with bone mineral density and positively associated with serum albumin (180). Our results are expanding these initial observation show a significant negative association between low bone density and Aix in a large population cohort. Moreover, we demonstrated that central blood pressure that was represented by central pulse pressure and Aix are more significantly associated with low BMD and fractures, in woman but also in younger strata of elderly men, not previously reported. This further indicates the strength of measuring central blood pressure.

D. Genetics of the HXB/BXH set

The recombinant inbred strains of rats were so far used to identify genetic variants that underlie cardiovascular traits among other diseases (127). Hypertension is polygenically inherited which makes the identification of its genes rather difficult. The SHRs were among the first model of rats used for genetic analyses in search of hypertensive genes (181). Pravenec et al. then carried their analysis on the RIS and found a significant association between blood pressure and the genes that were found in the RT1 complex (region of the major histocompatibility complex in rats) (127). Further studies done by Hamet et al., identified a restriction fragment length polymorphism in the hsp70 gene that is mapped onto the RT1 complex to be associated with 15 mmHg difference in blood pressure (182). These investigators demonstrated utility of RIS for longitudinal pnenotypic and genetic studies since all crosses of strains are fully inbred, homozygous at all loci (133). Multiple studies were carried afterwards in the aim of localising more loci that would be related to hypertension (144, 145, 183, 184). The RIS also served in identifying genes for metabolic syndrome, such as the cd36 and Srebp1 genes located on chromosomes 4 and 10 respectively. The cd36 was recognised to be responsible for encoding a transmembrane fatty acid transporter. The reduced expression of this gene in SHR was depicted to cause impairment of insulin activity, glucose intolerance and a decreased transport of fatty acids in adipose tissues and muscles (141, 185, 186). The Srebp1 was found to encode two isoforms of transcription factors Srebp1a and Srebp1c that are important in lipid regulation and carbohydrate metabolism (187). Further analysis carried out by Pravenec et al. found an association between the Srebp1 gene

and increased hepatic triglycerides and cholesterol but it did not seem to affect blood pressure (188). All those studies emphasise the importance of the recombinant inbred strains of rats in genetic studies, especially hypertension related ones. Different human genetic and animal studies were carried in search of genes that might be responsible for some traits seen in osteoporosis cases such as BMD, trabecular number etc... A whole linkage scan analysis carried by Shen et al. on 53 pedigrees that included 630 subjects, showed a significant linkage signal on chromosome X for the wrist and the hip BMD (189). Another study that was carried on the 1473 subjects from 323 pedigrees obtained from the Framingham Osteoporosis study showed a significant linkage of the femur shaft subperiostal width and chromosome X (190). Some other studies identified bone parameters such as cortical thickness and the femoral neck cross sectional geometry to be also associated or highly linked to the chromosome X (191, 192). Different other autosomes were identified to be associated with different bone parameters, among which is chromosome 1 that was found to be associated with volumetric BMD, bone surface, structural model index and trabecular volume (193-196). Our results showed a significant quantitative trait locus (QTL) on X chromosome for the trabecular bone volume trait. Spearman correlation analysis of BV/TV with the published phenotypes for HXB/BXH set (192 phenotypes) showed that the only blood pressure phenotype which correlated positively with BV/TV was the delta N^G-nitro-L-arginine-methyl ester (L-NAME) effect (mmHg), on 12 weeks of age males**.** BV/TV was associated with 3 significant markers when plotted with marker regression on X chromosome. On the other hand, Tb.N had several suggestive hits on X chromosome for marker regression

analyses. And the CIM revealed a suggestive interaction between chromosome 1 and X. The QTL for angiotensin converting enzyme 2 (ACE2) was located on chromosome X in three genetically hypertensive rats (197). In addition another study showed a significant association between the genetic variation of CAE 2 and left ventricular mass in women and a diminished ventricular function in men (198). Taken all together; data from our study suggests a possible link between blood pressure and bone parameters. Considering the QTLs for blood pressure on chromosome 1 and X that were identified by Huang et al. and Crackower et al. (142, 197) and the interaction that we found for trabecular number between chromosome 1 and X, which would imply a certain genetic influence between the two traits. Another possibility that can be drawn from our results and the research of others is a common link that can exist between BV/TV and the nitric oxide pathway, considering the correlation seen in our results of bone volume with the delta L-Name effect in 12 weeks of age male RIS. Nitric oxide is an important mediator of many physiological activities; it plays an important role on the endothelial cells and mediates vasodilation and it is implicated in bone formation as it regulates osteoblasts and osteoclasts activity (199, 200). Knockout animal models for endothelial NO were seen to have osteoporosis due to bone malformation (201, 202). The significant correlation that we observed between bone volume and the effect of L-Name, in addition to the interaction between chromosome 1 and X for the trabecular number trait, hints to the potential existence of a common genetic link between hypertension and low bone density seen in our rat model.

Conclusion

Both our studies point to the relation between hypertension and low bone mineral density. In CARTaGENE, we showed that hypertension was associated with a shorter stature in elderly, lower bone density and a higher rate of fractures compared to normotensives. In addition higher arterial stiffness was significantly associated with low bone density. Along the same line, we observed that hypertensive rats developed a low bone volume, insulin resistance and an increased calcium excretion while aging. Our genetic analysis suggested the presence of a link between genes bone volume and blood pressure. Further genetic and expression analysis are needed in order to refine our search and identify the specific genes involved.

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