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ASSOCIATION BETWEEN ADDUCIN-1 G460W VARIANT AND BLOOD PRESSURE IN SWEDES IS DEPENDENT ON INTERACTION WITH BMI AND SEX.

Short title: Adducin-1 and blood pressure in Swedes

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Abstract

Background. The W-allele of the G460W polymorphism in the Adducin-1 gene has been occasionally associated with increased blood pressure (BP). The aim of this study was to test if the G460W variant is associated with BP levels and BP progression rate and whether G460W associations with BP are affected by gender, body mass index (BMI) or age.

Methods. The G460W polymorphism was genotyped in the population based Malmö Diet and Cancer material - cardiovascular arm (MDC-CVA; n=6103), of whom 53% had also been examined 11±4.4 years earlier in the Malmö Preventive Project (MPP).

Results. Among subjects without antihypertensive treatment (AHT) at MDC-CVA (n=5009) there was no difference between carriers (38%) and non-carriers (62%) of the W-allele

in SBP (139.2±18.2 vs 139.2±18.5, P=0.99) or DBP (85.9±9.1 vs 86.1±9.2, P=0.49).

In subjects free from AHT at MPP and MDC (n=2637) there was no difference between carriers (38%) and non-carriers (62%) in progression of SBP (2.0±2.5 vs 2.0±2.7

mmHg/year, P=0.45) or DBP (0.59±1.6 vs 0.56±1.5 mmHg/year, P=0.66) from MPP to

MDC. At MDC-CVA BP was influenced by interaction between the G460W and BMI (P=0.02 for SBP and P=0.002 for DBP) and by interaction between G460W and sex

(P=0.03 for SBP and P=0.02 for DBP), a result further confirmed by stratified analysis

showing that female carriers of the W-allele belonging to the upper tertile of BMI had increased SBP (146.1±18.6 vs 141.2±18.6 mmHg; P<0.001), DBP (88.7±8.7 vs 86.1±8.7

mmHg; $P < 0.001$) and prevalence of hypertension (72.5 vs 61.8 %; $P = 0.001$).

Conclusion. Our data suggest that the G460W polymorphism influences blood pressure when BMI and gender are taken into account.

Key words: adducin-1, genetics, hypertension, blood pressure, kidney.

Introduction

High blood pressure even within the normal range is responsible for millions of deaths and disabilities throughout the world ^{1,2}. Blood pressure is a complex trait and genetic and environmental factors have a complex interrelation to determine individual blood pressure values³.

Adducin is a heterodimeric cytoskeletal protein highly conserved through the phylogenesis. Adducin promotes the organization of the spectrin-actin lattice by favoring its binding and controlling the rate of actin polymerization as an end capping actin protein ^{4,5}.

Adducin is formed by 3 subunits encoded by three genes (ADD-1, ADD-2 and ADD-3). These proteins and the genes encoding them have received attention as adducin polymorphisms have been shown to affect blood pressure both in rats and humans through an enhanced constitutive sodium re-absorption ⁶⁻¹⁰. In humans 2 polymorphisms of the ADD-1 gene lead to amino acid substitution: G460W and S586C⁷. Rat ADD-1 tyrosine at amino acid positions 316 and human tryptophane at amino acid positions 460, bind with higher affinity and activate the Na-K ATPase more strongly than the respective normal protein ¹¹.

Moreover, expression of the hypertensive rat 316Y or human 460W variant of adducin into normal renal epithelial cells recreates the hypertensive phenotype with higher Na-KATPase activity, $\mu 2$ -subunit hyperphosphorylation, and impaired Na-K-ATPase endocytosis¹².

A recent review reported that several linkage studies, exploring DNA markers very close to the ADD-1 locus and 18 out of 20 association studies taking into account variables reflecting body sodium or the renin-angiotensin system have shown positive results regarding the effect of the G460W variant of ADD-1 on blood pressure and hypertension. Furthermore, 12 out of 16 studies found that the ADD-1 polymorphism is associated with stroke, coronary artery disease, or renal and vascular dysfunction ¹³.

On the other hand there have been several studies in different populations, where no effect of the 460W allele on either blood pressure or hypertension has been found¹⁴⁻²² and in one study the G460 allele was associated with hypertension ²³.

Blood pressure is a complex phenotype and the effect of single gene variants is likely to be quite small. It is likely that many single gene variants can be detected only when other factors, genetic or environmental, allows them to be expressed ^{8,9,24-29}.

The aim of the present study was to test the possible association of the G460W polymorphism with cross-sectional blood pressure levels or blood pressure change over time and whether possible G460W associations with blood pressure are affected by gender, body mass index (BMI) or age.

Methods

The population studied was taken from the Malmö Diet and Cancer study (MDC) cohort³⁰. Blood pressure along with other cardiovascular risk factors was measured in a random sample of the MDC, referred to as the MDC cardiovascular arm (MDC-CVA) (n=6103). Successfully extracted genomic DNA in MDC-CVA, which was required for inclusion in the present study, was available on 6055 subjects but 50 people were excluded from the analysis because of unsuccessful genotyping (see below). The study of blood pressure as a continuous variable in MDC-CVA was restricted to subjects free from antihypertensive medication (n=5009) whereas also the patients on antihypertensive medication (n=996) were included when the dichotomized phenotype of “hypertension” and “normotension” was studied. Of the untreated subjects in MDC-CVA we were able to study the blood pressure change over time in 2637 subjects who previously had been investigated in another cohort study from Malmö - the Malmö Preventive project (MPP) and were free from antihypertensive medication also at MPP, which was performed approximately 10 years before MDC-CVA³¹.

Subjects followed from MPP to MDC-CVA, and who were free from antihypertensive treatment at both examinations (n=2637), had a mean “follow-up time” of 11.2 ± 4.4 years (range 1.0-19.3 years).

All study participants had given written informed consent. The Ethics Committee of the Medical Faculty of Lund University approved the study. The procedures were in accordance with the institutional guidelines.

Clinical characteristics of all subjects are shown in Table 1

Phenotyping

Blood pressure was measured twice by specially trained nurses in the right brachial artery in the supine position after 5 minutes rest using a mercury sphygmomanometer and the average of the two values was taken as the blood pressure. Korotkoff sounds corresponding to “phase I” was used to define the SBP and “phase V” the diastolic blood pressure (DBP). Blood pressure change over time from MPP to MDC-CVA was expressed as mmHg increase of blood pressure per year i.e. [follow - up time in years BP at MDC- CVA - BP at MPP]. In order to adjust for blood pressure values at baseline, the blood pressure change over time was also expressed as percent increase in blood pressure per year i.e. [follow - up time in years

BP at MDC- CVA - BP at MPP / BP at MPP x 100]. Hypertension (was defined as being on antihypertensive treatment or having SBP or DBP $\geq 140/90$ mmHg according to current diagnostic criteria and normotension as having SBP and DBP $<140/90$ mmHg³². BMI was calculated as the ratio of the weight in kilograms to the square of the height in meters (kg/m^2).

Genetic tests.

DNA was extracted from frozen granulocyte or buffy coat samples using QIAamp-96 spin blood kits (QIAGEN, VWR Sweden) at the DNA extraction facility supported by SWEGENE. The G460W SNP in the ADD-1 gene [dbSNP accession number “rs4961”] was genotyped. Genotyping was performed using the ABI 7900 (Applied Biosystems, Foster City, California, USA) using

Forward primer: 5'-GAGAAGACAAGATGGCTGAACTCT -3',

Reverse primer: 5'- GTCTTCGACTTGGGACTGCTT-3',

synthesized by Applied Biosystems. TaqMan MGB probes were custom synthesized by Applied Biosystems: wild-type (A): G460 probe: 5'-VIC- CATTCTGCCCTTCCTC -3', and Mutant W460 probe: 5'-FAM- ATTCTGCCATTCCTC -3'.

PCRs were run in the TaqMan Universal Master mix (Applied Biosystems, Foster City, California, USA), according to manufacturer recommendations. The TaqMan assay plates were transferred to a Prism 7900HT instrument (Applied Biosystems) in which the fluorescence intensity in each well of the plate was read. Fluorescence data files from each plate were analyzed using automated software (SDS 2.1; Applied Biosystems) ³³.

Statistics

All data were analyzed with SPSS statistical software (version 12.0.1, SPSS Inc. Chicago, Illinois, USA). Frequency differences were analyzed by χ^2 -test. Continuous variables are presented as mean \pm standard deviation (SD). Significance of differences in continuous variables was tested by t-test. Multiple linear regression and multiple logistic regression analysis were used in the multivariate models with blood pressure and hypertension status, respectively, as dependent variables and genotype, age, sex and BMI as independent variables. Multiple linear regression analysis was used also to test for interaction of genotype and either age, sex and BMI regarding effect on blood pressure. All tests were two-sided and throughout $P < 0.05$ was considered statistically significant.

Results

In the total material (n=6055), the genotyping success rate was 99.2% (n=6005) including patients on antihypertensive medication. Of the successfully genotyped subjects 3772 (62.8%) were homozygotes for the “wild type” G-allele, 1983 were heterozygotes (33.0%) and 250 (4.2%) were homozygotes for the variant W-allele. This finding is in line with previous reports in Caucasian subjects¹³.

Genotype distributions, in all groups of subjects studied, were in accordance with Hardy-Weinberg equilibrium (data not shown).

Blood pressure at MDC-CVA and blood pressure change over time from MPP to MDCCVA

were similar in all genotype groups (Figure 1). In subjects who were normotensive at MPP, there was no difference in the proportion of subjects who converted from normotension to hypertension from MPP to MDC-CVA between carriers of 460W (37.9%) and carriers of the wild type genotype (38.0%) ($P=0.98$). Adjustments for age, sex and BMI, which were independently related to both SBP and DBP, did not change any of these results (data not shown).

At MDC-CVA, blood pressure was influenced by significant interaction between the G460W and BMI as well as by interaction between G460W and sex (tables 2-3), indicating that the 460W variant together with female sex and 460W variant together with increasing BMI is associated with higher systolic and diastolic blood pressure.

A stratified analysis of the data confirmed that subjects in the upper tertile of BMI (BMI > 26.65 Kg/m²) expressing the W-allele had higher SBP and DBP as compared to non carriers (145.4 \pm 18 vs 143.4 \pm 18.2; $p=0.03$ for SBP and 90.0 \pm 9.0 vs 88.3 \pm 9.1, $P<0.001$ for DBP, figure 2). Further stratification for sex showed that the association was present only in females in the upper tertile of BMI (146.1 \pm 18.6 vs 141.2 \pm 18.6, $p<0.001$ for SBP and

88.7±8.7 vs 86.1±8.7, $p<0.001$ for DBP, figure 2).

In the analysis of the dichotomous variable of hypertension/normotension at MDC-CVA, thereby allowing inclusion also of subjects on antihypertensive medication who were excluded in the analyses of the continuous phenotype of blood pressure, there was no difference in the prevalence of the W-allele in hypertensive patients as compared to normotensive subjects (37.1% vs 37.4%, $p=0.78$). Similar to the continuous variables of SBP and DBP, logistic regression revealed that hypertension prevalence was influenced by significant interaction between the G460W and BMI as well as by interaction between G460W and sex (table 4), indicating that the 460W variant together with female sex and 460W variant together with increasing BMI is associated with higher prevalence of hypertension.

Stratified analysis showed significantly higher prevalence of the W-allele in hypertensive overweight patients as compared to normotensive subjects (table 5). Again, the significant association was present in females only (table 5).

Discussion

We found that there is no overall association between the G460W polymorphism and blood pressure in a large Swedish population based sample. However, there was significant interaction between the G460W polymorphism and both sex and BMI regarding blood pressure and hypertension prevalence.

The kidney plays a major role in blood pressure homeostasis and hypertension development and functional variants of genes that interfere with sodium reabsorption are attractive candidate genes. Bianchi and coworkers have focused a lot of attention on ADD-1, a cytoskeletal protein that has been shown to augment the activity of the Na-K pump. The Na-K pump is an ATPase that drives the reabsorption of sodium in the kidney but it is present and active in several other tissues, including vascular smooth muscle cells (VSMC) ¹³. An enhanced activity of the Na-K pump in the SMC could putatively lead to enhanced vasodilatation with diminished peripheral resistance and lower blood pressure. Thus, it is possible that the effect of the functional G460W polymorphism, enhancing the Na-K pump activity in the kidney with consequent augmented sodium handling, under different conditions, could be blurred or even counteracted from the effect elicited in other tissues such as VSMC. Thus, most studies controlling for body sodium or renin-

angiotensin-aldosterone system (RAAS) activity have found positive associations between the W-allele and higher blood pressure¹³. Also in our sample we could not find simple association of the G460W polymorphism with blood pressure but when the interaction of this genetic variant with sex and BMI was considered a positive association resulted evident both for SBP and DBP, suggesting that the effect of the polymorphism is unmasked when these 2 well established blood pressure covariates are taken into account ¹⁴⁻²². A stratified analysis of the data confirmed that subjects with BMI in the upper tertile and expressing the W-allele have higher SBP, DBP and hypertension prevalence respect to wild type carriers. Interestingly, the effect of the W-allele was particularly evident in females. It is possible that lack of or even reversed associations between G460W and blood pressure when significant cofactors are not taken into account are due to the single gene effect of G460W being diluted by the polygenic background of blood pressure, assuming that the

G460W primarily affects the sodium sensitive component of blood pressure. In addition, one could speculate that the importance of the renal versus the VSMC effects of the G460W is likely to be relatively greater in subjects with endothelial dysfunction as the latter could be expected to blunt the vasodilatory effects of the W-allele in SMC but not the enhanced sodium transport induced by W-allele in renal tubular cells.

Endothelial dysfunction as well as salt sensitivity have been associated with both increasing age and obesity³⁴⁻³⁸. Regarding gender it has to be underlined that in our sample women are mostly in post-menopause (Table 1), which has been found associated both with salt sensitivity³⁹ and endothelial dysfunction⁴⁰.

Previous studies have shown that homozygosity for the W-allele is significantly associated with reduced renal plasma flow and glomerular filtration rate as compared with the wildtype

variant⁴¹ and that acute changes in body Na⁺ may differently affect blood pressure in humans as a function of alpha-adducin genotype⁹.

All these effects could be difficult to detect at the general population level where other counterbalancing effects may be active. Interaction and stratified analysis can help to unravel the biological complexity of genetic variants that act in concert with other factors. The importance of interaction analyses in the interpretation of single gene effects in complex traits such as blood pressure is further emphasized by the fact that the G460W polymorphism was negatively associated with blood pressure when the interaction terms of genotype/sex and genotype/BMI were simultaneously taken into account. Genetic and environmental heterogeneity of blood pressure homeostasis and essential hypertension development have made it difficult to repeatedly link genetic variants to blood pressure and hypertension in humans. Among genes that exert their roles in sodium handling, ADD-1 is one of the most attractive candidates but at the general population level its effect has not invariably been confirmed in previous studies. On the other hand only minor effects are expected from a single SNP:s and these effects could be blurred from complex gene-gene and gene-environment interaction. Studies of adequate size are needed to analyze if the effects of these genetic variants could be exacerbated by other factors such as obesity or gender. Thus major efforts are needed in future in order to specifically define and characterize more homogeneous subgroups of people where specific genetic variants could have a primary role in determining blood pressure levels.

In conclusion we have found that the G460W polymorphism doesn't play a major role either in blood pressure or hypertension development in the general population when possible interactions are disregarded. Positive interaction of G460W with sex and BMI and the result of the stratified analysis suggest that this polymorphism could be of primary importance for blood pressure homeostasis and hypertension prevalence in obese females probably joined by a salt-sensitive background. Further studies of adequate size are needed to confirm our findings and to explore the hypothesis that salt sensitivity could be the factor subtending the effect of the G460W polymorphism of the ADD-1 gene on BP at the general population level.

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Table 1. Anthropometric and metabolic features of the subjects investigated in the “Malmö Diet and cancer-cardiovascular arm” and “Malmö Preventive Project”.

Variables	MPP to MDC-CVA without AHT (N=2637)	MDC-CVA without AHT (N=5009)	MDC-CVA with AHT (N=996)
Gender male, %	54.9	41.8	44.7
Age, years	47.0 ± 5.7	57.1±5.9	59.3 ± 5.6
BMI, Kg/m²	24.1 ± 3	25.5±3.8	27.5 ± 4.5
SBP, mmHg	122.4±13.2	139.2±18.3	152.3 ± 19.4
DBP, mmHg	81.9 ± 8.4	86.0±9.1	92.0 ± 9.6
ΔSBP, mmHg/year	2.0±2.7		
ΔDBP, mmHg/year	0.58±1.54		
ΔSBP, %/year	1.7±2.3		
ΔDBP, %/year	0.77±2.1		

Data are presented either as mean ± standard deviation or percentage.

MPP, Malmö Preventive project; MDC-CVA , Malmö Diet and Cancer; BMI, body mass index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; ΔSBP, Delta Systolic Blood Pressure; ΔDBP, Delta Diastolic Blood Pressure.

Table 2. Linear regression analysis of Systolic Blood Pressure at MDC-CVA (n=5002*).

Variables	Regression Coefficient	Standard Error	Standardized Coefficient	95% CI of regression coefficient	P
<i>Intercept</i>	70.62	3.21		64.32/76.92	<0.001
Gender †	-3.33	0.61	-0.09	-4.53/-2.13	<0.001
Age, year	0.93	0.04	0.30	0.85/1.07	<0.001
BMI, Kg/m²	0.82	0.08	0.17	0.66/0.97	<0.001
ADD-1_G460W ‡	-11.95	5.91	-0.31	-19.61/-4.29	0.002
ADD-1_G460W‡ x SEX §	2.27	1.01	0.10	0.30/4.25	0.024
ADD-1_G460W‡ x BMI§	0.33	0.13	0.23	0.07/0.59	0.013
Model					<0.001

BMI, body mass index; ADD1 adducin 1; MDC-CVA , Malmö Diet and Cancer-cardiovascular arm; C.I., confidence interval.

* N.B. Seven subjects were not included in the analysis due to missed BMI

† male sex is coded as 1 and female sex as 2.

‡ For the ADD-1_G460W polymorphism the G-allele is coded as 0 and the W-carrier as 1.

§ The statistical variables used for the interaction (ADD-1_G460W x SEX, ADD-1_G460W x BMI) have been computed by multiplying the ADD-1_G460W genotype respectively with sex and BMI.

The interaction terms ADD-1_G460W‡ x AGE and ADD-1_G460W‡ x BMI § x SEX were discarded from the regression model because not significant

Table 3. Linear regression analysis of Diastolic Blood Pressure at MDC-CVA (n=5002*).

Variables	Regression Coefficient	Standard Error	Standardized Coefficient	95% C.I. of regression coefficient	P
<i>Intercept</i>	70.16	1.65		67.16/74.42	<0.001
Gender †	-3.22	0.32	-0.17	-3.84/-2.60	<0.001
Age, year	0.14	0.02	0.09	0.10/0.18	<0.001
BMI, Kg/m²	0.50	0.04	0.21	0.42/0.58	<0.001
ADD-1_G460W ‡	-18.5	5.11	-0.98	-28.49/-8.46	<0.001
ADD-1_G460W‡ x SEX §	8.09	2.91	0.73	2.38/13.8	0.006
ADD-1_G460W‡ x BMI §	0.66	0.19	0.90	0.27/1.04	0.001
ADD-1_G460W‡ x BMI § x SEX §	-0.27	0.11	-0.62	-0.48/-0.049	0.016
Model					<0.001

BMI, body mass index; ADD1 adducin 1; MDC-CVA , Malmö Diet and Cancer-cardiovascular arm; C.I., confidence interval

* N.B. Seven subjects were not included in the analysis due to missed BMI.

† male sex is coded as 1 and female sex as 2.

‡ For the ADD-1_G460W polymorphism the G-allele is coded as 0 and the W-carrier as 1.

§ The statistical variables used for the interaction (ADD-1_G460W x SEX, ADD-1_G460W x BMI, ADD-1_G460W‡ x BMI § x SEX §) have been computed by multiplying the ADD-1_G460W genotype respectively with sex, BMI and sex multiplied for BMI.

The interaction term ADD-1_G460W‡ x AGE was discarded from the regression model because not significant

Table 4. Logistic regression analysis of hypertension prevalence at MDC-CVA (n=5002*).

Variables	Regression Coefficient	Standard Error	O.R.	95% CI	P
<i>Intercept</i>	-6.78	0.38	0.001		<0.001
Gender †	-0.46	0.07	0.63	0.55/0.73	<0.001
Age, year	0.08	0.005	1.09	1.08/1.10	<0.001
BMI, Kg/m²	0.11	0.01	1.12	1.10/1.14	<0.001
ADD-1_G460W ‡	-4.16	1.34	0.016	0.001/0.22	0.002
ADD-1_G460W‡ x SEX §	1.89	0.76	6.6	1.50/29.1	0.013
ADD-1_G460W‡ x BMI §	0.14	0.052	1.16	1.043/1.28	0.006
ADD-1_G460W‡ x BMI § x SEX §	-0.062	0.030	0.94	0.89/0.99	0.036
Model					<0.001

BMI, body mass index; ADD1 adducin 1; MDC-CVA , Malmö Diet and Cancer-cardiovascular arm, C.I., confidence interval , O.R., Odds ratio

* N.B. Seven subjects were not included in the analysis due to missed BMI.

† male sex is coded as 1 and female sex as 2.

‡ For the ADD-1_G460W polymorphism the G-allele is coded as 0 and the W-carrier as 1.

§ The statistical variables used for the interaction (ADD-1_G460W x SEX, ADD-1_G460W x BMI, ADD-1_G460W‡ x BMI § x SEX §) have been computed by multiplying the ADD-1_G460W genotype respectively with sex, BMI and sex multiplied for BMI.

The interaction term ADD-1_G460W‡ x AGE was discarded from the regression model because not significant

Table 5. Distribution of normotensive and hypertensive subjects at MDC-CVA according to genotype and after stratification for sex and BMI (tertiles) (n=5998).

BMI (tertiles)		All (N=5998*)		P- value	Male subjects (N=2540)		P- value	Female subjects (N=3465)		P- value
		G460G (N=1277)	W-carriers (N=719)		G460G (N=600)	W-carriers (N=349)		G460G (N=677)	W-carriers (N=370)	
BMI ≥27.00 (N=1996)	Hyp	969 (62.5)	581 (37.5)	0.012	495 (63.2)	288 (36.8)	0.99	474 (61.8)	293 (38.2)	0.001
	Nor	308 (69.1)	138 (30.9)		105 (63.3)	61 (36.7)		203 (72.5)	77 (27.5)	
		G460G (N=1258)	W-carriers (N=795)		G460G (N=620)	W-carriers (N=346)		G460G (N=638)	W-carriers (N=398)	
BMI ≥23.91 <27.00 (N=2002)	Hyp	795 (63.8)	451 (36.2)	0.25	407 (65.6)	213 (34.4)	0.21	388 (62.0)	238 (38.0)	0.75
	Nor	463 (61.2)	293 (38.8)		213 (61.6)	133 (38.4)		250 (61.0)	160 (39.0)	
		G460G (N=1235)	W-carriers (N=765)		G460G (N=383)	W-carriers (N=237)		G460G (N=852)	W-carriers (N=528)	
BMI <23.91 (N=2000)	Hyp	644 (62.8)	382 (37.2)	0.36	223 (64.3)	124 (35.7)	0.16	421 (62.0)	258 (38.0)	0.87
	Nor	591 (60.7)	383 (39.3)		160 (58.6)	113 (42.4)		431 (61.5)	270 (38.5)	

Data are presented as absolute number of subjects (percentage of subjects).

MDC-CVA , Malmö Diet and Cancer-cardiovascular arm; Hyp, Hypertensive subjects; Nor, normotensive subjects.

N.B. 7 subjects were not included in the analysis because of missing value of BMI.

Titles and legends to figures.

Legend figure 1

Systolic and Diastolic Blood pressure values at MDC-CVA (a and b) and Systolic and Diastolic Blood pressure change from MPP to MDC-CVA (c and d) according to genotypes and after stratification for sex. (n=5009)

G460G White bar,

460W Black bar

MDC-CVA, Malmö Diet and Cancer-cardiovascular arm; MPP, Malmö Preventive Project; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; n.s. not significant

Legend figure 2

Systolic and Diastolic Blood pressure values at MDC-CVA according to genotypes in females (a and c; n=2912) and males (b and d; n=2090) after stratification for BMI (tertiles).

G460G White bar

460W Black bar

* N.B. 7 subjects were not included in the analysis because of missing value of BMI.

MDC-CVA , Malmö Diet and Cancer-cardiovascular arm; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BMI, Body Mass Index; n.s. not significant.

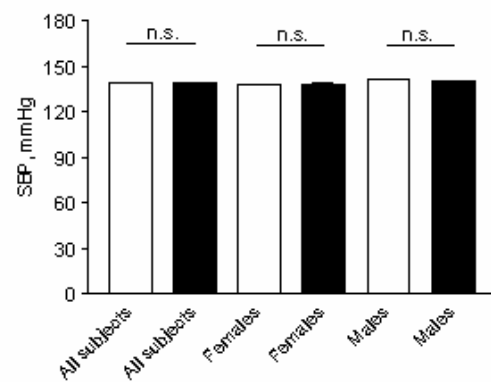
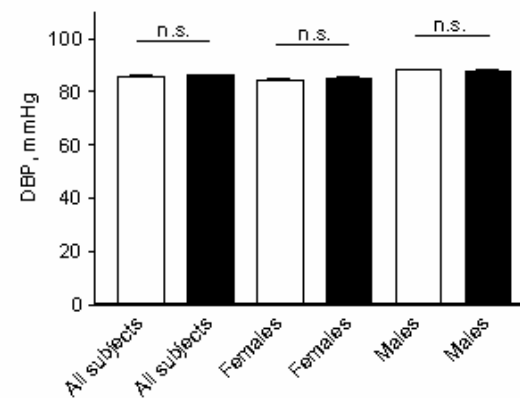
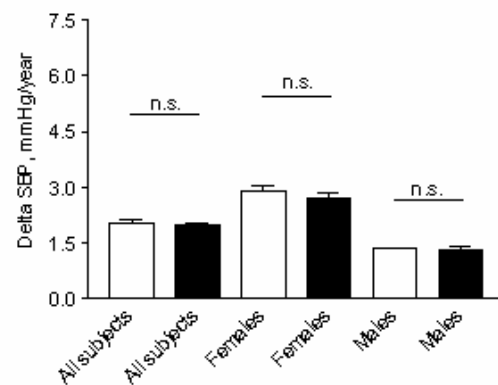
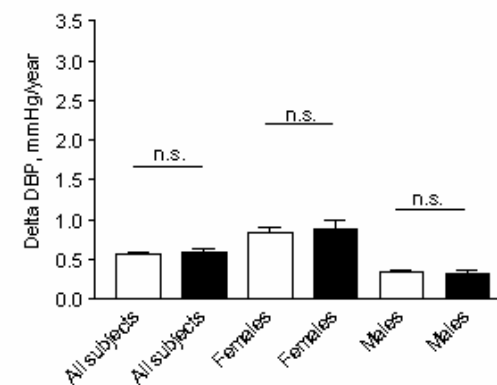
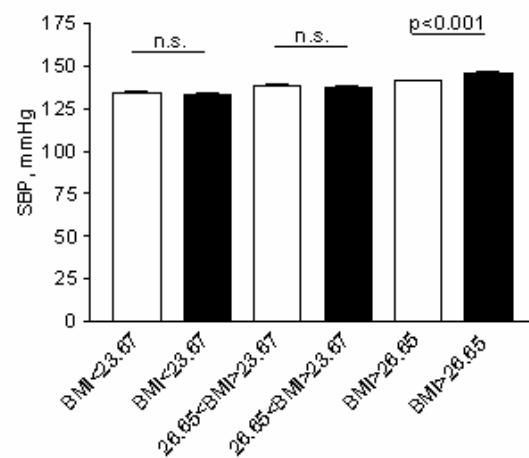
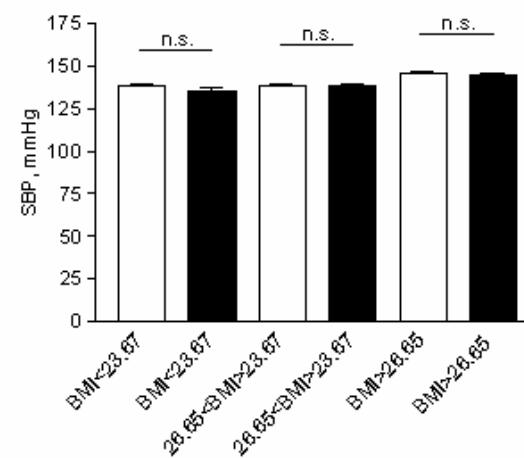
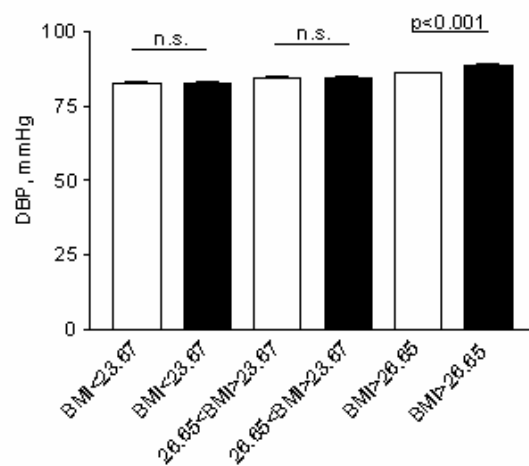
Figure 1**a****b****c****d**

Figure 2**a****b****c****d**