

**Homocysteine Metabolism**  
**5<sup>th</sup> International Conference**  
**Milano (Italy), June 26-30, 2005**  
**Guest Editor: Marco Cattaneo**

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# Homocysteine Metabolism

5<sup>th</sup> International Conference  
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Guest Editor: *Marco Cattaneo*

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## ORAL COMMUNICATIONS PLENARY SESSIONS

### PLENARY SESSION 4 COGNITIVE IMPAIRMENT

#### S001

##### THE EFFECT OF 3-YEAR FOLIC ACID SUPPLEMENTATION ON COGNITIVE FUNCTION. A RANDOMIZED CONTROLLED TRIAL

Durga J, van Boxtel MPJ, Schouten EG, Jolles J, Kok FJ, Verhoef P

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**Background.** Observational studies show that low levels of folate are associated with poor cognitive performance. Randomized controlled trials conducted thus far have not lead to convincing evidence to support or refute a causal role of folate in cognitive decline. Interpretation is hampered by the fact that these trials were either too small, too short, or did not properly assess cognitive performance.

**Aims.** To determine whether 3-year daily folic acid supplementation decreases or even reverse cognitive decline associated with aging.

**Methods.** We conducted a double blind randomized placebo-controlled single center study in 818 men and postmenopausal women, aged 50-70 years, with plasma concentrations of total homocysteine  $\geq 13$  mmol/L and serum vitamin B12  $\geq 200$  pmol/L at screening. Subjects were recruited from municipal and blood bank registries in the Netherlands. Participants received either 0.8 mg folic acid as an oral supplement or placebo following randomization. The main outcome of the study was performance in different domains of cognitive function: sensorimotor speed, cognitive flexibility and verbal memory (as compound measures) and information processing speed and word fluency. These functions were assessed at the start and at the end of the study using a battery of neurocognitive tests.

**Results.** The results of the trial will be presented at the conference.

### PLENARY SESSION 6 EMERGING ASSOCIATIONS

#### S002

##### PLASMA TOTAL HOMOCYSTEINE AND BONE MINERAL DENSITY. THE HORDALAND HEALTH STUDY

Gjesdal CG, Vollset SE, Ueland PM, Refsum H, Drevon CA, Tell GS

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**Background.** Plasma total homocysteine (tHcy) has been associated with hip fracture in observational studies, but it is uncertain to what extent this association is mediated by low B-vitamin status or by lowered bone mineral density (BMD).

**Aims.** We examined the association of BMD of the hip with plasma tHcy, folate and vitamin B12.

**Methods.** BMD of total hip was measured in 2268 men and 3070 women, aged 47-50 and 71-75 years, from the Hordaland Homocysteine Study cohort. Osteoporosis was investigated in the elderly participants and was defined as T-score  $< -2.5$ . Linear and logistic regression models were used, adjusting for BMI, smoking status, plasma creatinine concentration, coffee consumption, level of physical activity and (for women) current use of postmenopausal estrogen.

**Results.** Plasma levels of tHcy were inversely related to BMD in middle-aged and elderly women ( $p < 0.001$ ), but not in men. The multiple adjusted odds ratio for osteoporosis among subjects with high ( $> 15$  micromol/L) compared to low ( $< 9$  micromol/L) tHcy was 2.8 (95% CI: 1.6-5.0) for elderly women, and not significant for elderly men. Additional adjustments for plasma folate or intake of calcium and vitamin D did not alter the results. Plasma folate was associated with BMD in elderly women only. We observed no association between BMD and vitamin B12.

**Conclusion.** Elevated tHcy and low folate were associated with reduced BMD and increased risk of osteoporosis of the hip in women but not in men. These findings suggest that tHcy may be a potential modifiable risk factor for osteoporosis in women.



## ORAL COMMUNICATIONS WORKSHOPS

### WORKSHOP 1

#### COGNITIVE IMPAIRMENT and OTHER NEURO-PSYCHIATRIC DISEASES

##### C001

##### LOW PLASMA METHIONINE, CYSTEINE, AND GLUTATHIONE LEVELS ARE ASSOCIATED WITH INCREASED FREQUENCY OF COMMON POLYMORPHISMS AFFECTING TRANSMETHYLATION AND TRANSSULFURATION PATHWAYS IN CHILDREN WITH AUTISM

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Autism is a complex neurodevelopmental disorder with a reported prevalence of 1 in 1000 children in the US. Although both genetic and environmental factors are thought to be involved, none has been reproducibly identified. The metabolic basis for autism has received much less research attention despite the fact that chronic biochemical imbalance is often a primary factor in the development of complex disease. The metabolic phenotype provides a window through which the interactive impact of genes and environment may be viewed and relevant susceptibility factors identified. Although abnormal thiol metabolism has been associated with other neurologic disorders, these pathways and related polymorphisms have not been evaluated in autistic children. In this study, metabolites in methionine transmethylation and transsulfuration pathways were measured in plasma from 95 autistic and 75 control children using HPLC with electrochemical detection. Common polymorphic variants in genes coding for transcobalamin II (TCII), methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR), catechol-O-methyltransferase (COMT), and glutathione-S-transferase (GST) M1 and T1 were subsequently evaluated in 360 autistic children and 205 controls. The results of the metabolic study indicated that mean plasma levels of methionine, the ratio of S-adenosylmethionine to S-adenosylhomocysteine, cysteine, total glutathione, and the ratio of reduced to oxidized glutathione (redox ratio) were significantly decreased among the autistic children relative to age-matched controls. Multiple regression analysis was used to create a metabolic equation that is able to predict the presence of autism with high accuracy. In the genotype analysis, the frequency of MTHFR 677CT/1298AG, TCII 776GG, COMT 1947GG, and the GST M1 null genotypes were increased in the autistic children relative to controls, whereas the MTRR G allele was decreased. We hypothesize that an increased vulnerability to oxidative stress (environmental and/or intracellular) may contribute to the development and clinical manifestations of autism.

##### C002

##### VITAMIN B12, HOLOTRANSCOBALAMIN AND RISK OF COGNITIVE IMPAIRMENT IN OLDER PEOPLE

Clarke R,<sup>1</sup> Birks J,<sup>2</sup> Hin H,<sup>3</sup> Emmens K,<sup>1</sup> Nexo E,<sup>4</sup> Schneede J,<sup>5</sup> Ueland PM,<sup>6</sup> Sherliker P,<sup>1</sup> Lewington S,<sup>1</sup> Halsey J,<sup>1</sup> Molloy AM,<sup>7</sup> Scott J,<sup>7</sup> Grimley Evans J<sup>2</sup>

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**Background and aims.** The importance of vitamin B12 (B12) deficiency for cognitive impairment may have been underestimated due to the limitations of the current B12 assays. We examined the associations of cognitive impairment with total cobalamin, holotranscobalamin ([holoTC], the metabolically-active fraction of B12) and metabolic markers of B12 deficiency, methylmalonic acid (MMA) and homocysteine (tHcy) in two population-based studies of older people living in Oxford, England.

**Methods.** A combined cross-sectional analysis involving 2278 people (1276 from the Oxford Healthy Aging Population [OHAP] and 1002 from Banbury) examined the associations of cognitive impairment defined by a Mini-Mental State Examination score <22/30 with quartiles of B12 status. Logistic regression was used to examine these associations after adjustment for age, sex and study.

**Results.** The mean age (SD) for OHAP was 77.6 (6.4) and for Banbury study population was 81.4 (4.6) years. After adjustment for age, sex and study, cognitive impairment was inversely associated with levels of holoTC and positively associated with MMA and tHcy, but not with B12.

**Table 1. Odds ratios (& 95%CI) of cognitive impairment adjusted for age, sex and study.**

Quartiles of	I	II	III	IV	p (trend)
HoloTC	2.0 (1.3-3.1)	1.4 (0.9-2.1)	1.5 (0.9-2.4)	1.0	p=0.003
B12	1.0 (0.7-1.5)	0.8 (0.5-1.2)	0.9 (0.6-1.3)	1.0	p=0.92
tHcy	1.0	1.0 (0.6-1.5)	1.4 (0.9-2.1)	1.5 (1.0-2.3)	p=0.02
MMA	1.0	1.1 (0.7-1.8)	1.1 (0.7-1.9)	2.1 (1.3-3.3)	p=0.003

**Conclusions.** The associations of B12 status with cognitive impairment were strong and graded and present not just at low levels, but also at levels previously considered normal. Large trials of B12 supplements in older populations are required to assess the clinical relevance, if any, of these associations.

## C003

**COGNITIVE PERFORMANCE IN VITAMIN B12 DEFICIENT VEGAN MEN WITH INTERMEDIATE HYPERHOMOCYSTEINAEMIA**Bell V,<sup>1</sup> Lloyd-Wright Z,<sup>2</sup> Moller J,<sup>3</sup> Hvas AM,<sup>4</sup> Nexø E,<sup>4</sup> Sanders TAB<sup>2</sup><sup>1</sup>School of Psychology, Cardiff University; <sup>2</sup>Nutrition Food & Health Research Centre, King's College London; <sup>3</sup>Department of Clinical Biochemistry, Aarhus University Hospital at Skejby; <sup>4</sup>Department of Clinical Biochemistry, AKH, Aarhus University Hospital

**Background.** Previous studies examining cognitive function in relation to vitamin B12 status have shown mixed results. The strongest evidence for a link appears in two sub-groups of the population: the healthy elderly and people with dementia. However, the lack of research on healthy participants makes the relationship between B12 and cognitive function difficult to gauge in the general population. One population of interest are vegans, who can be subject to deprivation of vitamin B12 through their elimination of animal products.

**Aims.** To assess the effect of vitamin B12 supplementation on cognitive performance in a sample of healthy vegan men (n=138, mean age 48, range 18-80) using robust neuropsychological tasks with the necessary sensitivity to detect sub-clinical effects.

**Methods.** Vegan participants (n=39) with serum vitamin B12 levels <120 ng/L were given the active supplement, 18 of this subgroup undertook structural MRI scans before supplementation. Vegan participants with serum levels of 120-200 ng/L (n=35), were given either active or placebo treatment. A subgroup of vegan participants with serum vitamin B12 >200 ng/L (n=64) were used as controls. Homocysteine (tHcy) and methylmalonic acid (MMA) were measured using isotope dilution GCMS with SIM. Holotranscobalamin (holoTC) was measured by ELISA, folate and serum vitamin B12 by a competitive protein binding immunoassay.

**Results.** There were no significant differences in cognition between controls and other participants. Despite intermediate hyperhomocysteinaemia, reversed by vitamin B12 supplementation, there were virtually no significant effects of supplementation on the cognitive tests. Adequate power was present to accept the null hypothesis. Structural MRI showed no detectable neuropathology.

**Conclusions.** Supplementation of this population with long term vitamin B12 deficiency, did not improve cognitive performance; this was attributed to the protective effects of high folate and reduced saturated fat intake of vegans.

## C004

**PLASMA HOMOCYSTEINE LEVELS, MTHFR POLYMORPHISMS 677C>T, 1298A>C, 1793G>A, AND SCHOOL ACHIEVMENT IN A POPULATION SAMPLE OF SWEDISH CHILDREN**Borjel AK,<sup>1,2</sup> Nilsson TK,<sup>1,2</sup> Hurtig Wennlof A,<sup>2,3</sup> Yngve A,<sup>3</sup> Sjöström M<sup>3</sup><sup>1</sup>Department of Clinical Chemistry, Örebro University Hospital, Örebro; <sup>2</sup>Department of Caring Sciences/Biomedicine, Örebro University, Örebro; <sup>3</sup>Unit of Preventive Nutrition, Karolinska Institute, Huddinge Hospital, Stockholm Sweden

**Background.** Many studies have found a link between impaired Hcy metabolism and neuropsychiatric disorders such as depression, dementia, and cognitive impairment in the elderly.

**Aims.** Our aim was to study the plasma Hcy levels in school children in relation to MTHFR and TCII polymorphisms, and to investigate whether Hcy levels and the polymorphisms are related to school achievement, a neurocognitive function.

**Methods.** 692 Swedish school children aged 9 or 15 years were genotyped for the polymorphisms.

**Results.** The MTHFR 677C>T polymorphism was associated with increased tHcy levels in both age groups ( $p < 0.001$ ) in both sexes, whereas the 1298A>C polymorphism was significantly and inversely related to plasma tHcy levels in the 15-year-olds ( $p = 0.004$ ), as was also the 1793G>A polymorphism ( $p < 0.001$ ). TCII776C>G was unrelated to Hcy. Both total sum of school grades and sum of grades in 10 core subjects were significantly and negatively associated with plasma Hcy levels ( $p = 0.005$  and  $p = 0.009$ , respectively). In multiple regression analyses when Hcy and MTHFR 677C>T genotype were both independent variables, Hcy was significantly and negatively related to school grades and there was also a trend for MTHFR.

**Conclusions.** We interpret this as an MTHFR 677TT-dependent gene-environment interaction upon plasma homocysteine levels which has significant impact on neurocognitive function in Swedish school children.

## C005

**HOMOCYSTEINE AND FOLATE ARE ASSOCIATED WITH DIFFERENT DOMAINS OF COGNITIVE FUNCTION IN OLDER ADULTS**Miller JW, Ramos MI, Green R  
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**Background.** Hyperhomocysteinemia is associated with cognitive impairment and dementia in the elderly. Folate deficiency, a primary cause of hyperhomocysteinemia, is also associated with cognitive impairment and dementia, and it is often assumed that the influence of folate deficiency on brain function is mediated solely through homocysteine. However, associations between homocysteine and cognitive function remain significant after controlling for confounding by folate, and vice versa. This suggests that homocysteine and folate affect brain function through different mechanisms.

**Aims.** To compare the associations between folate, homocysteine, and cognitive function in a cross-sectional cohort of older adults ("60y; n=1789).

**Methods.** Global cognitive function was assessed by the modified mini-mental state exam (3MSE) and six specific subdomains of cognitive function by cross-culturally validated neuropsychological tests. RBC folate was determined by automated chemiluminescence and total plasma homocysteine by HPLC.

**Results.** After controlling for confounding by demographic variables, vitamin B12, creatinine, and depressive symptoms, RBC folate was directly associated ( $p = 0.005$ ) and homocysteine was inversely associated ( $p = 0.02$ ) with 3MSE, the test of global cognitive function. RBC folate was also directly associated with delayed recall ( $p = 0.007$ ), a test of episodic memory. In contrast, homocysteine was not associated with delayed recall, but was inversely associated with three other cognitive function tests, picture association ( $p = 0.05$ ), verbal attention ( $p = 0.04$ ), and pattern recognition ( $p = 0.001$ ). These tests primarily assess domains of cognitive function other than memory.

**Conclusions.** Folate and homocysteine are associated with different domains of cognitive function in older adults, suggesting that folate deficiency and hyperhomocysteinemia independently influence brain function, likely through different mechanisms.

**Funding.** NIH AG12975 and AG10129, and USDA 00-35200-9073.

## C006

**PLASMA HOMOCYSTEINE IS CORRELATED WITH BRAIN ATROPHY AND WHITE MATTER INJURY IN ALZHEIMER'S DISEASE**

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**Objective.** To investigate the relationship of plasma homocysteine (Hcy) to white matter disease (WMD) and brain atrophy in Alzheimer's Disease (AD)/Mild Cognitive Impairment (MCI) and cerebral amyloid angiopathy (CAA).

**Background.** Accumulating data link Hcy, radiographic measures of ischemia or neurodegeneration, and cognitive impairment in healthy elderly individuals. Little data are available regarding neuroimaging correlates of Hcy in AD/MCI and the related condition of cerebrovascular amyloid deposition or CAA.

**Methods.** Plasma Hcy, folate, vitamin B12 and creatinine levels were determined in 39 subjects with AD, 21 with MCI, and 49 with CAA-related intracerebral hemorrhage. A rater blinded to clinical and laboratory information graded MRI scans for cortical atrophy (CAtr), ventricle/brain ratio (VBR) as a measure of subcortical atrophy, and periventricular (PVH) and subcortical (SWMH) white matter hyperintensities using validated scales from the Rotterdam Scan Study. Cortical atrophy (range: 0-15) and PVH (range: 0-9) were assessed semi-quantitatively whereas SWMH and VBR were quantitatively measured on axial MRI images.

**Results.** In AD/MCI patients Hcy was strongly correlated with CAtr ( $r=0.65$ ,  $p<0.001$ ) and moderately with PVH ( $r=0.41$ ,  $p=0.001$ ), SWMH ( $r=0.39$ ,  $p=0.002$ ) and VBR ( $r=0.47$ ,  $p<0.001$ ). These associations persisted when adjusted for age, creatinine, folate, vitamin B12, and history of hypertension or heart disease. Among CAA subjects, Hcy correlated with VBR ( $r=0.43$ ,  $p=0.002$ ) and to a lesser extent with CAtr ( $r=0.27$ ,  $p=0.063$ ) but not with PVH ( $r=0.11$ ,  $p=0.43$ ) or SWMH ( $r=0.2$ ,  $p=0.15$ ). CAA subjects had lower Hcy ( $p=0.03$ ), but more PVH ( $p=0.002$ ) and SWMH ( $p=0.001$ ) than AD/MCI.

**Conclusions.** Hcy is associated with WMD and atrophy in AD. The relationship with WMD does not persist in the presence of advanced CAA.

## C007

**HOMOCYSTEINE AND COGNITIVE DECLINE IN NORMAL OLDER ADULTS: TIME FRAME OF CHANGES IN FOCUSED ATTENTION AND MEMORY**

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High homocysteine (tHcy) is a known risk factor for dementia in older adults. We have examined the time course and relationship between changes in tHcy levels, focused attention and memory tests scores.

Participants ( $n=115$ ) 65 years of age or older had normal cognitive function at baseline. Follow-up occurred at 2.3 and 5 years. At every visit, psychometric tests (Stroop Neuropsychological Screening Test or Stroop, and Mattis Dementia Rating Scale or DRS) and blood for biochemical determinations was obtained. Participants were divided according to changes in their tHcy from baseline to follow-up as: Group 1- increased tHcy, Group 2- decreased tHcy, and Group 3- unchanged tHcy. Paired T-test was used to compare scores at baseline and 5 years follow-up.

**Results.** Age, years of education, and MMSE score were similar between groups at baseline and follow-up. Subjects whose tHcy increased from baseline to year 5 ( $p<0.0005$ ) showed a decrease in Stroop score (90.7 to 82.6,  $p<0.0005$ ), DRS memory sub-score (24.1 to 22.5,  $p<0.0005$ ) and DRS total (139.3 to 137.9,  $p<0.05$ ). Subjects whose tHcy decreased ( $p<0.001$ ) did not have significant decline in their scores (Stroop 82.4 to 83.3, DRS memory 24.3 to 23.3, and DRS total 139.0 to 138.3). Subjects whose tHcy did not change showed a decrease in Stroop score (84.4 to 76.1,  $p<0.0005$ ) and DRS memory (24.4 to 23.7,  $p<0.0005$ ). Results at the 2.3 years follow-up showed a significant inverse correlation between increases in tHcy and decrease in Stroop scores ( $p<0.001$ ), but not between increases in tHcy and decline in memory scores.

**Conclusions.** These results suggest: 1. Increasing tHcy levels over time are related to a decrease of focused attention (determined by significant declines in the Stroop score) and of memory scores in older adults, and 2. Declines in focused attention seem to precede memory decline.

## C008

**PLASMA HOMOCYSTEINE LEVEL IS PREDICTIVE OF LATER COGNITIVE IMPAIRMENT IN COMMUNITY-DWELLING OLDER ADULTS**

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**Background.** Higher plasma total homocysteine (tHcy) levels have been shown to be independently associated with poorer cognitive performance in older adults with and without Alzheimer's disease.

**Aims.** To determine the associations of tHcy levels with decline from normal cognitive function to cognitive impairment.

**Methods.** A group of cognitively healthy controls was followed up for 6 years after baseline measures of cognitive performance and blood factors associated with dementia were obtained. Cognitive Impairment (CI) at follow-up was defined using clinical criteria for MCI (Mild Cognitive Impairment) and dementia. Follow-up measures for subjects 70 years and older were available for 58 controls and 37 cognitively impaired subjects. Logistic regression analyses were used to determine whether baseline tHcy tertiles were predictive for group (control or CI) at follow-up with covariates age, further education, sex and Apo-E4 status entered.

**Results.** Groups were not different in terms of age, sex, Apo-E status or years of further education. Test performance was worse in the CI group in domains of episodic, semantic and working memory, processing speed and executive function. tHcy levels were raised in the CI group as compared with the control group at baseline ( $14.2\pm 4.2$  micromoles/L vs.  $12.1\pm 2.8$  micromoles/L,  $p<0.011$ ). Group at follow-up was predicted by tHcy tertiles, so that the risk of being in the CI group was greater for those in the top tertile of tHcy versus those in the lowest tertile at baseline (OR: 3.54, 95% Confidence Intervals: 1.21-10.3). At follow-up there was also a cross-sectional association between tHcy tertiles and CI (OR 3.38, 1.15-9.87).

**Conclusions.** Raised tHcy levels at baseline were predictive of cognitive impairment as assessed by cognitive tests at follow-up and by clinical conversion to MCI or dementia.

## WORKSHOP 2

### DETERMINANTS of HOMOCYSTEINE

C009

#### THE CREMONA HOMOCYSTEINE STUDY

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A population study was conducted in the area of Cremona (Lombardia, Italy) during the years 1990-91, mainly focused on estimating the prevalence of diabetes mellitus and impaired carbohydrate tolerance. Great attention was also given to the collection of information regarding clinical and laboratory markers of cardiovascular risk. A sample of 3,599 subjects aged 40-87 years was identified. They were invited to attend a medical visit in three outpatient clinics over a period of 3 months. Subjects were submitted to blood sampling fasting and after oral glucose load (75 g) and then visited by attending physicians. Biochemical evaluations included: blood glucose at baseline and 2 hours after the glucose load, insulinemia, fibrinogen, total cholesterol, HDL cholesterol, triglyceride, Lp(a), tHcy, folate, vitamin B12 (IMX, Abbott), PLP (home-made RIA) and cystatin-C (Analyzer II, Behring). 2,066 subjects participated in the study (58% of invited subjects) with no apparent selection for sex and age. Familiarity for diabetes showed some minor influence on participation, but no evidence of a selection bias was apparent for any additional characteristics.

In March 1998 information was gained regarding causes of death occurring for each subject since January 1991. 41% of the variation in plasma levels of fasting tHcy was explained by gender, folate, cystatin C, age, alcohol intake, vitamin B12 and triglyceride levels. 159 subjects (7.7%) died during the 7-year follow up, mainly of cardiovascular diseases or cancer. In a Cox model adjusted for gender, age and other relevant risk factors, tHcy levels were independent predictors of all-cause mortality. While the risk of cardiovascular mortality increased gradually across the quartiles of the tHcy distribution, only subjects in the fourth quartile of the tHcy distribution suffered increased cancer mortality. These data show that in a rural-industrial area of Italy, homocysteine is an independent risk factor for cardiovascular and cancer mortality.

C010

#### A GENOME-WIDE LINKAGE SCAN FOR HOMOCYSTEINE LEVELS IDENTIFIES THREE REGIONS OF INTEREST

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A high homocysteine level is a risk factor for many clinical conditions, including venous thrombosis. Homocysteine levels are under control of genetic and environmental factors. Extensive candidate gene studies have identified genetic

variants that influence homocysteine levels, including the MTHFR 677C>T polymorphism, but so far only a part of the genetic variation in homocysteine can be explained. In order to identify chromosomal regions that influence the plasma homocysteine level without the necessity of advanced knowledge of candidate genes a genome-wide linkage analysis was conducted. A datafile consisting of 13 pedigrees and 469 subjects with homocysteine measurements was available. A set of 377 markers covering the genome was genotyped in 275 subjects. Heritability was estimated and twopoint and multipoint linkage analyses were performed using the variance component linkage method (SOLAR version 2.1.3). The heritability of the age and sex adjusted homocysteine levels was 44% ( $p=7.7 \times 10^{-11}$ ). One region with suggestive linkage with homocysteine levels was identified on chromosome 16 (LOD score 1.76; nominal  $p=0.0024$ ). Further, indications of linkage with regions on chromosome 12 (LOD score 1.57; nominal  $p=0.0036$ ) and chromosome 13 (LOD score 1.52; nominal  $p=0.0041$ ) were found. The homocysteine level in our dataset was highly heritable. The genetic linkage analysis identified three regions that showed weak to suggestive evidence of linkage with plasma homocysteine levels.

C011

#### BETAINE AS DETERMINANT OF FASTING PLASMA HOMOCYSTEINE: EFFECT IS MODIFIED BY FOLATE STATUS AND MTHFR 677C->T GENOTYPE

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**Background.** Plasma betaine is a strong determinant of the increase in plasma total homocysteine (tHcy) after oral methionine loading, and a weaker determinant of fasting tHcy levels. Previous data indicate that the betaine effect is more pronounced at low folate. The TT genotype of the MTHFR 677C□ T polymorphism predisposes to mild hyperhomocysteinemia.

**Aims.** To assess the combined influence of folate status and MTHFR 677C□ T genotype on betaine as determinant of fasting plasma tHcy.

**Methods.** Samples from 2556 healthy subjects aged 50-65 of both genders, selected from the NORCCAP (Norwegian Colorectal Cancer Prevention) cohort (n=10700) according to MTHFR 677C□ T genotype and equally distributed between the three genotypes, were analysed for betaine and B-vitamins.

**Results.** Median concentrations of homocysteine were higher, and betaine and folate lower, in the TT compared to the other genotypes. Betaine showed a moderate correlation to homocysteine in the TT, and a weak relation in the CT group. In subjects with low folate (<9.7 nanomol/L) and the MTHFR 677 TT genotype, betaine in the lowest quartile was associated with 5.4 micromol/l higher tHcy, as compared to the highest betaine quartile, after multiple adjustments. In the CT and CC groups, the increase was 1.7 micromol/L ( $p<0.05$ ) and 1.0 micromol/L (ns), respectively. In subjects with folate levels above 13.5 nanomol/L, betaine had only a minor influence on plasma tHcy.

**Conclusions.** Betaine is a strong determinant of plasma tHcy in subjects with low folate status, predominantly in those with the TT genotype of the MTHFR 677C□ T polymorphism.

## C012

**HOMOCYSTEINE, VITAMINS AND DIETARY LIFE FACTORS IN EUROPEAN ELDERLY: THE SENECA STUDY**

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The SENECA study started in 1988 and consisted of a random age- and sex-stratified sample of inhabitants of 19 European towns. A total of 2199 elderly people (1,072 men, 73±1.8 yr at baseline and 1,103 and 1109 females, 73±1.8 yr at baseline) participated. Lowest values for tHcy corresponded to Mediterranean countries (Portugal, Spain, and Greece), compared to central or northern European countries (Netherlands or Belgium (differences higher than 4 µmol/L). For SENECA Finale an interesting north-south gradient is observed, with the lowest values for tHcy corresponding to Betanzos (Spain), 12.38 µmol/L followed by both centers in Portugal, whereas the highest concentrations are found in Maki (Poland), 21.92 µmol/L and Culemborg (Netherlands), 20.41 µmol/L, with a mean tHcy concentration for all the centers of 15.98 µmol/L. Effect of sex has been also evaluated: those countries with the lowest tHcy concentration (i.e. Spain or Portugal) show significant ( $p<0.01$ ) higher tHcy concentration in men vs. women, whereas these differences by sex are not observed in countries with the highest tHcy values. The effect of *aging* (Baseline vs. Finale) was also evaluated, with no differences observed for the same individuals in the 10-years period. Plasma folic acid was compared to Hcy values, resulting also in marked differences between north and southern countries. Plasma vitamin B12 also shows a close pattern. Neither albumin nor total cholesterol, HDL-cholesterol or triglycerides were associated with tHcy. By contrast, total alcohol intake was positively and significantly ( $p<0.01$ ) correlated with tHcy (no intake corresponded with the lowest tHcy, 14.3 µmol/L vs. high intake-over 30 g/d-with the highest tHcy, 17 µmol/L). The type of alcoholic beverage was also evaluated: wine and spirits drinkers showed positively significant ( $p<0.005$ ) correlation whereas beer intake was not significantly associated. Smoking was also analysed: *never* smokers had the lowest tHcy concentration (13,82±0,20 µmol/L) vs. *current* smokers (16,64±0,35), a significant difference ( $p<0,05$ ).

## C013

**INFLUENCE OF THE ENOS GENE POLYMORPHISMS ON THE HOMOCYSTEINE PLASMA LEVELS IN HEALTHY SUBJECTS**Sofi F, Fatini C, Sticchi E, Marcucci R, Rossi L, Prisco D, Casini A,<sup>1</sup> Surrenti C,<sup>1</sup> Gensini GF, Abbate R*Department of Medical and Surgical Critical Care, Thrombosis Centre, University of Florence; Azienda Ospedaliera-Università Careggi, Florence; <sup>1</sup>Department of Clinical Pathophysiology, Unit of Clinical Nutrition, University of Florence, Italy*

Nitric oxide (NO) plays a relevant role in various events during atherogenesis. *In vitro* data suggested that NO may modulate plasma homocysteine (Hcy). Aim of this study was to investigate the role of endothelial nitric oxide synthase (eNOS) T-786C, G894T and 4a/4b polymorphisms in influencing Hcy plasma levels. Blood samples were obtained from 1287 unrelated subjects. Hcy plasma levels were determined by fluorimetric polarized immuno assay, folate and vitamin B12 levels by radio immuno assay, vitamin B6 by

high performance liquid chromatographic assay and eNOS and MTHFR polymorphisms by polymerase chain reaction-restriction fragment length polymorphism analysis. MTHFR C677T polymorphism significantly influenced Hcy levels after adjustment for all confounding variables ( $p$  for trend  $<0.0001$ ). Univariate analysis showed that eNOS T-786C but not G894T and 4a4b polymorphism was significantly associated with the risk of having Hcy in the 3rd tertile ( $>13.4$  µmol/L) (OR: 1.2 95%CI 1.02-1.5;  $p=0.03$ ).

After adjustment for all variables known to influence Hcy levels, T-786C polymorphism still remains to influence Hcy levels (OR: 1.9 95%CI 1.1-3.2;  $p=0.01$ ). By analysing the influence of eNOS polymorphisms on Hcy plasma levels according to vitamin levels (folate, vitamin B6 and vitamin B12), age ( $<60$  yrs), and smoking habit we showed a significant association between the eNOS T-786C polymorphisms and Hcy levels in non smoker subjects with normal vitamin status, aged less than 60 yrs. In conclusion, we documented that eNOS T-786C, but not G894T and 4a4b polymorphisms, mildly but significantly influences Hcy plasma levels.

## C014

**POSITIVE ASSOCIATION BETWEEN PLASMA CONCENTRATIONS OF HOMOCYSTEINE AND SYMMETRIC AND ASYMMETRIC DIMETHYLARGININE IN THE GENERAL POPULATION. THE HOORN STUDY**

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*Background and aims.* Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) and a risk factor for endothelial dysfunction and cardiovascular disease. The related compound symmetric dimethylarginine (SDMA) does not inhibit NOS, but may limit nitric oxide production by competing with arginine for cellular uptake. Because both methyl groups of ADMA and SDMA are derived from methionine, the synthesis of both compounds is accompanied by generation of two equivalents of homocysteine. The present study was undertaken to investigate whether plasma concentrations of total homocysteine (tHcy) are associated with concentrations of ADMA and SDMA.

*Methods.* The study was performed in 366 men and 360 women, aged 50 to 75, who participated in the Hoorn study, a population-based cohort study. ADMA, SDMA and tHcy were measured by HPLC. Glomerular filtration rate (GFR) was estimated by the simplified MDRD equation.

*Results.* The median (interquartile range) concentration of tHcy was 11.7 (9.5-14.7) µmol/L. ADMA concentrations (µmol/L) increased across quartiles of tHcy (from 0.48±0.06 for Q1 to 0.52±0.08 for Q4;  $p<0.0001$  for trend). This trend was also observed for SDMA concentrations (0.47±0.07 for Q1 to 0.61±0.18 for Q4;  $p<0.0001$  for trend). In a multivariate linear regression model, adjusted for age, sex, GFR and glucose tolerance status, both ADMA and SDMA were independently associated with log-transformed tHcy concentrations. Regression coefficients (percent increase of tHcy per 1 SD increment of methylated arginine) were 4.0 (95% CI 1.2-6.8;  $p=0.004$ ) for ADMA and 6.3 (95% CI 2.-9.9;  $p=0.0003$ ) for SDMA.

*Conclusions.* Both ADMA and SDMA are independently associated with plasma tHcy. This association is consistent with the hypothesis that part of the cardiovascular risk associated with elevated tHcy is mediated by inhibition of the nitric oxide pathway by methylated arginines.

## C015

**B-VITAMINS AS DETERMINANTS OF PLASMA HOMOCYSTEINE IN SUBJECTS WITH DIFFERENT MTHFR 677C->T GENOTYPES**

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**Background.** B-vitamins partly determine plasma concentrations of total homocysteine (tHcy). The MTHFR 677C->T polymorphism, which is an important genetic tHcy determinant in the general population, modifies the effects of folate and riboflavin.

**Aims.** To assess the effect of several B-vitamins as determinants of plasma tHcy in relation to the MTHFR 677C->T polymorphism.

**Methods.** 2546 men and women aged 50-65 years were selected from the NORCCAP (Norwegian Colorectal Cancer Prevention) cohort (n=10700) according to MTHFR genotype and equally distributed between the CC, CT and TT groups. Blood samples were analysed by LC-MS/MS (tHcy, riboflavin, betaine, PLP and creatinine in EDTA-plasma) and microbiological methods (folate and cobalamin in serum). Data were analysed by multiple regression. Genotype effects were assessed by interaction analyses and data stratification.

**Results.** Mean concentrations of plasma tHcy were 10.0  $\mu\text{mol/l}$ , 10.6  $\mu\text{mol/l}$  and 13.2  $\mu\text{mol/l}$  in subjects with the CC, CT and TT genotypes, respectively. Sex, age, folate, riboflavin, betaine, PLP, cobalamin, creatinine and the MTHFR 677C->T polymorphism were related to plasma tHcy in multivariate models ( $p < 0.001$ ), and B-vitamin and betaine effects were strongest in subjects with the T allele ( $p < 0.004$ ). In the TT group, the estimated tHcy difference between subjects with concentrations of serum folate in the lowest compared to the highest quartile was 4.9  $\mu\text{mol/l}$ . The corresponding tHcy difference was 3.9  $\mu\text{mol/l}$  for riboflavin, 3.2  $\mu\text{mol/l}$  for betaine, 3.1  $\mu\text{mol/l}$  for PLP and 2.7  $\mu\text{mol/l}$  for cobalamin.

**Conclusions.** Several B-vitamins and betaine were robustly related to plasma concentrations of tHcy in middle-aged subjects selected from a population-based cohort. The MTHFR 677C->T polymorphism profoundly modified micronutrient effects.

## C016

**DIET AND FOOD GROUP ASSOCIATIONS WITH PLASMA TOTAL HOMOCYSTEINE LEVEL: THE HORDALAND HOMOCYSTEINE STUDY**

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**Background.** There are few large studies on diet and food groups as predictors of concentrations of total homocysteine (tHcy) in plasma.

**Aims.** The objective was to examine associations between a variety of food groups and plasma tHcy in a general population not using vitamin supplements.

**Methods.** The study population included 2441 of 47-49 and 2370 of 71-74 year-old men and women from the second round of the Hordaland Homocysteine Study (1997-99)

who did not use vitamin supplements. The subjects completed a food-frequency questionnaire and provided blood samples for biochemical analysis. Multiple linear regression analyses were used adjusted for age and sex.

**Results.** We observed statistically significant differences in plasma tHcy across increasing quartiles of the various food groups. There were negative associations between plasma tHcy and the intake of bread, cereals, vegetables, fruits, fish, eggs and milk. The estimated difference in tHcy level per increasing quartile of food intake was -0.13 (95% CI: -0.24, -0.03)  $\mu\text{mol/l}$  for the intake of bread, -0.20(-0.31,-0.10)  $\mu\text{mol/l}$  for cereals, -0.33(-0.43,-0.24)  $\mu\text{mol/l}$  for vegetables, -0.30(-0.39,-0.20)  $\mu\text{mol/l}$  for fruits, -0.23(-0.32,-0.13)  $\mu\text{mol/l}$  for fish, -0.23(-0.32, -0.13)  $\mu\text{mol/l}$  for eggs, and -0.14 (-0.23, -0.04)  $\mu\text{mol/l}$  for milk. A positive association was seen for sweets, 0.18 (0.08, 0.28)  $\mu\text{mol/l}$  per increasing quartile. No significant associations were seen for potatoes, cheese, meat and fat.

**Conclusions.** In a Norwegian adult and elderly population sample not taking vitamin supplements, plasma level of tHcy is associated with diet, especially with the intake of food groups such as bread, cereals, vegetables, fruits, fish, eggs, milk and sweets, all of which (except sweets) were associated with lower tHcy level.

## WORKSHOP 3

**CARDIOVASCULAR and THROMBOTIC DISEASES**

## C017

**DIETARY FOLATE INTAKE AND RISK OF FUTURE MYOCARDIAL INFARCTION: RESULTS FROM THE EPIC-POTSDAM-COHORT**

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Prospective data on folic acid/folate intake and risk of cardiovascular disease is inconsistent and mainly limited to American studies where intake of synthetic folic acid is much higher than in most European countries due to the considerably higher use of supplements and consumption of fortified foods. The present investigation aims to assess the relation between dietary folate equivalents intake (DFE) and risk of myocardial infarction (MI) in the German EPIC-Potsdam cohort which is part of the multi-centre European prospective investigation into cancer and nutrition study. In Potsdam, about 27,000 healthy men and women between 35 and 65 years were recruited between 1994-98. During an average follow-up time of 4.6 years 129 MI cases were verified among 22,245 non-users of vitamin-supplements without prevalent ischemic heart disease at baseline. Participants were divided according to median DFE intake (103  $\mu\text{g}$ ), assessed by a validated food frequency questionnaire. Compared to intake of DFE below the median, higher intake was associated with a Hazard ratio (HR) of 0.63 (95% confidence interval (95% CI) 0.44-0.90) in an age and sex-adjusted Cox proportional hazards model. Adjustment for established dietary and cardiovascular risk factors altered the HR to 0.57 (95% CI 0.36-0.91). Stratified analysis according to ethanol intake below/above the median (8.1 g/d) showed that the

inverse association between DFE intake and risk of myocardial infarction was most pronounced in those with ethanol intake above the median (HR=0.39, 95% CI 0.22-0.68) and attenuated in those with an ethanol intake below the median (HR=0.75, 95% CI 0.41-1.36), using participants of low DFE intake and low ethanol intake as the reference.

In conclusion, increased DFE intake was observed to be associated with decreased risk of myocardial infarction in a German study population pointing towards the importance of folate intake with respect to primary prevention of cardiovascular disease.

## C018

### LOW PLASMA LEVELS OF VITAMIN B6 AND RECURRENT VENOUS THROMBOSIS: RISK ASSESSMENT AND EFFECT OF COMBINED VITAMIN SUPPLEMENTATION IN THE VITRO-TRIAL

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**Background.** Low vitamin B6 levels in plasma have been reported to be a risk factor for venous thromboembolism (VTE) independently of homocysteine concentration in case-control and prospective studies (Cattaneo *et al*, 2001 and 2003).

**Aims.** We tested the hypothesis that a possible protective effect of combined supplementation with folate, vitamin B12 and vitamin B6 on the risk of VTE might be explained not only by a decrease in homocysteine, but also by the restoration of normal plasma vitamin B6 levels.

**Methods.** We measured the baseline plasma levels of pyridoxal-L-phosphate (PLP), the coenzyme form of vitamin B6, in 701 patients with previous episodes of VTE who participated in the VITRO-study, a placebo-controlled, randomized clinical trial on the effect of daily oral administration of folate 5 mg, vitamin B12 0.4 mg and vitamin B6 50 mg. We assessed the risk of recurrent VTE for subjects with low PLP levels. Furthermore, we stratified the effect of combined vitamin supplementation in patients with low PLP plasma levels.

**Results.** A total of 93 VTE recurrences were reported (13.3%) during the 2.5 year of the study follow-up. Baseline PLP levels lower than the 10th percentile (<15.7 nmol/L) were associated with a 2.4 increased risk for recurrent VTE in the placebo group (95% CI 1.2-4.9,  $p=0.02$ ), but not in the vitamin group (RR=1.1 [95% CI 0.4-2.9],  $p=0.8$ ). Combined vitamin supplementation decreased the risk of VTE recurrences by 45% ( $p=0.3$ ) in the low PLP group (RR=0.56 [0.19-1.69]), compared to 8% in the group with normal PLP levels (RR=0.92 [0.59-1.42]).

**Conclusions.** This study confirms that low plasma PLP levels are a risk factor for recurrent VTE. The observed risk reduction for recurrent VTE accomplished by combined supplementation with folate, vitamin B12 and vitamin B6 could be (at least partly) explained by the restoration of normal vitamin B6 levels.

## C019

### LOW FASTING METHIONINE CONCENTRATION AS A NOVEL RISK FACTOR FOR RECURRENT VENOUS THROMBOSIS?

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Hyperhomocysteinemia is a risk factor for venous thrombosis. However, the mechanism through which homocysteine exerts its thrombogenic effect is unclear. One hypothesis is that the thiol-group of homocysteine interferes with components of the clotting system. If this is true, we expect that high cysteine is also a risk factor for venous thrombosis because it also has a thiol-group. Another hypothesis is that hyperhomocysteinemia reflects a disturbed methylation with a decreased supply of methylgroups, needed in methylation reactions. If this were true, low methionine would be expected as a risk factor. With these hypotheses in mind we performed a case-control study to investigate several components of the homocysteine metabolism for an association with venous thrombosis. We determined concentrations of methionine, homocysteine, cysteine, betaine, folate in plasma and folate in red blood cells in 185 patients with recurrent venous thrombosis and in 500 control subjects and assessed the associated thrombotic risk. Low methionine was associated with a gradual increased risk for recurrent venous thrombosis with a highest odds ratio of 3.4 (95% CI 2.0-6.0) for a methionine concentration below 20.6  $\mu\text{mol/L}$  (bottom quartile). Low methionine remained a risk factor [OR= 3.7 (95% CI 2.1-6.5)] for recurrent venous thrombosis after adjusting for homocysteine and cysteine, whereas the thrombotic risk associated with homocysteine [OR= 0.9 (95% CI 0.5-1.7)] was absent for each of the upper three quartiles. Cysteine yielded a highest odds ratio of 1.8 (95% CI 1.0-3.5) for the top quartile after adjustment. In conclusion, low fasting methionine concentration is associated with an increased risk on recurrent venous thrombosis. This risk association was stronger for methionine than for homocysteine and cysteine. This observation supports the hypothesis that a decreased supply of methylgroups for methylation reactions might be the pathogenic mechanism involved in the development of venous thrombosis in hyperhomocysteinemia.

## C020

### HOMOCYSTEINE AND AORTIC STIFFNESS IN MEN A POPULATION BASED CROSS SECTIONAL STUDY

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Experimental evidence suggests mild to moderate hyperhomocysteinemia as an independent risk factor for cardiovascular disease. The proatherosclerotic effect of homocysteine is exerted either through a direct effect on the arterial wall or indirectly by involvement of oxidative stress pathway. Hyperhomocysteinemia may cause vascular changes by several mechanisms: through positive relation with blood pressure, endothelial dysfunction, and arterial stiffness. Some studies showed a strong correlation between homocysteine and arterial stiffness in subjects with elevated cardiovascular risk, including hypertension, diabetes mellitus,

and end-stage renal failure; however, this has not been observed consistently in relatively healthy subjects. We hypothesized that homocysteine is an independent marker of the presence and severity of the aortic stiffness in relatively healthy men, measured by carotid-femoral PWV. A population-based cross-sectional study was performed among 376 men, aged 40-80, randomly sampled of population of Utrecht, The Netherlands. Participants health information was obtained by medical history, standardized questionnaires including lifestyle factors and diet and physical examination. Aortic stiffness was assessed by measurement of the carotid-femoral pulse wave velocity (PWV) using applanation tonometry (Sphygmocor). Fasting plasma total homocysteine (tHcy) concentration was measured using Fluorescence Polarization Immunoassay. Data were analyzed by using multiple linear regression analyses; adjusted for age, mean arterial pressure, heart rate, wrist/hip ratio (model A). More adjustment has been done for diabetes mellitus, and smoking (model B). Overall, in adjusted multiple linear regression analyses we did not find a significant association between plasma tHcy and carotid-femoral PWV ( $r=0.040$ ; 95% CI  $-0.065$ ;  $0.145$ ). Excluding 53 men with prevalent CVD (14%), the association remained almost the same ( $r=0.04$ ; 95% CI  $-0.010$ ;  $0.096$ ). The interaction term for prevalent cardiovascular disease and tHcy was not significant ( $p=0.55$ ). This study does not support the evidence of an independent association between aortic stiffness and plasma total homocysteine in relatively healthy men.

**Table 1. The relation between plasma tHcy and PWV, expressed by multiply-adjusted regression coefficients.**

	Plasma tHcy linear	P-value	Plasma tHcy quartile <sup>†</sup>	P-value
	R (95% CI)		R (95% CI)	
Model A	0.053 (-0.011 to 0.117)	0.07	0.046 (-0.059 to 0.151)	0.07
Model B	0.047 (-0.017 to 0.112)	0.10	0.040 (-0.065 to 0.145)	0.09

Model A adjusted for age, mean arterial pressure, heart rate, wrist/hip ratio; Model B adjusted for age, mean arterial pressure, heart rate, wrist/hip ratio, diabetes, and smoking; Highest quartile versus lowest quartile of plasma tHcy concentration.

**C021**

**EFFECT OF HOMOCYSTEINE LOWERING THERAPY ON CORONARY ENDOTHELIAL FUNCTION IN PATIENTS WITH STABLE CORONARY ARTERY DISEASE. A SUB-STUDY OF THE WESTERN NORWAY B-VITAMIN INTERVENTION TRIAL (WENBIT)**

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**Background.** Total homocysteine (tHcy) is an independent risk factor for occlusive vascular disease and is also associated with endothelial dysfunction, an early marker of atherosclerosis.

**Aims.** We tested the effects of tHcy lowering therapy used in WENBIT on coronary endothelial function in patients with established coronary artery disease (CAD).

**Methods.** Forty patients (median age 57) undergoing elective percutan coronary intervention were randomised into four groups to daily oral treatment with a combination of 0.8 mg folic acid and 0.4 mg vitamin B-12 or placebo, and with 40 mg vitamin B-6 or placebo, in a 2x2 factorial design. At baseline and after 9 months of treatment, coronary endothelial function was assessed by coronary angiography and doppler flow-wire during incremental (0.72, 7.2 and 36.0 mikrogram/min) intracoronary doses of acetylcholine.

**Results.** At baseline, we found median tHcy 10.4 mikroM,

creatinine 87 mikroM, cholesterol 4.5 mmol/L and CRP 1.6 mg/L. Maximum flow achieved by acetylcholine correlated significantly to CRP ( $r=0.32$ ,  $p=0.047$ ), but no significant correlations were found between intracoronary flow parameters and plasma concentrations of tHcy, folate or vitamin B-6. In a linear regression model including age, tHcy, creatinine, total cholesterol, CRP, folate and vitamin B-6 as predictors of maximum flow, CRP was even stronger related ( $\beta=0.39$ ,  $p=0.015$ ). This relation was attenuated after 9 months ( $\beta=0.09$ ,  $p=0.6$ ). Treatment with folic acid and vitamin B-12 or vitamin B-6 did not influence change in coronary artery diameter, increase in average peak blood velocity (APV) or maximum flow achieved by intracoronary acetylcholine infusion.

**Conclusions.** Among patients with established CAD, 9 months of treatment with folic acid/B-12 or with vitamin B-6, given in addition to standard medical treatment, had no significant effect on endothelial mediated coronary tonus or flow. Inflammation is related to endothelial function, but this relation may be attenuated by continued medical therapy.

**C022**

**ROLE OF CLASSICAL RISK FACTORS AND HOMOCYSTEINE IN THE FAILURE OF WELL CONDUCTED WARFARIN THERAPY TO PREVENT ISCHEMIC COMPLICATIONS IN HIGH RISK PATIENTS WITH ATRIAL FIBRILLATION**

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**Background.** In patients with atrial fibrillation (AF) oral anti-coagulant therapy (OAT) is efficacious in reducing stroke and embolism. However, despite OAT, ischemic events do occur in some patients. Studies specifically addressing the identification of risk factors for ischemic events during well-conducted OAT are not available.

**Aims.** We report the results of a prospective study in AF patients on OAT followed by anticoagulation clinic on the role of classical risk factors and homocysteine for ischemic complications.

**Methods.** We prospectively observed 364 AF patients [mean age  $73.3 \pm 8.2$  years, 859 patient-years (pt/yr)].

**Results.** During follow-up time spent within, above and below the intended therapeutic range was 70%, 14% and 16% respectively. Eight major bleeding events (rate  $0.9 \times 100 \text{pt/yr}$ ) and 21 ischemic complications were recorded. No differences were observed in relation to the quality of OAT between these patients and all other patients. Patients who developed ischemic events during OAT had a higher prevalence of arterial hypertension (OR 4.5, 1.3-15.6 95% CI  $p=0.01$ ) and of history of previous ischemic event (OR 7, 2.3-21.4 95% CI  $p=0.001$ ) respect to the other patients. Homocysteine plasma levels were significantly higher in patients who developed ischemic events during OAT respect to patients without ( $22.3 \pm 11.8$  vs  $15.8 \pm 6.2$  micromol/L  $p=0.01$ ). Homocysteine levels higher than 95° percentile of our population ( $>29.8$  micromol/L) was significantly more represented in patients who developed ischemic events during OAT respect to the other patients (OR 6.4; 1.8-22.6 95% CI  $p=0.003$ ). The contemporary presence of these risk factors markedly increased the risk of the occurrence of ischemic events during OAT (OR 17.9; 4.1-78.3 95% CI  $p=0.000$ ).

**Conclusions.** In conclusion, we found that the above mentioned risk factors for ischemic events allow to identify a group of AF patients at high risk of ischemic complications during a well conducted OAT.

C023

**HOMOCYSTEINE AND MYOCARDIAL INFARCTION IN THE PROSPECTIVE EPIC POTSDAM COHORT**

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Data on the importance of homocysteine for cardiovascular disease (CVD) differ among retrospective and prospective studies. Retrospective studies show a stronger association between elevated homocysteine and CVD than studies with a prospective design. EPIC Potsdam is part of the multi-centre European prospective investigation into cancer and nutrition study. In Potsdam, about 27,000 healthy men and women between 35 and 65 years were recruited between 1994-98. Follow up at the end of April 2004 resulted in 157 verified cases of myocardial infarction. For the purpose of the present study, a control group of 850 subjects was drawn who are representative for the entire cohort.

In these subjects, plasma homocysteine, folate, cobalamin, pyridoxal-5-phosphate, and creatinine was measured. The MTHFR C677T polymorphism was also determined. The association between homocysteine and myocardial infarction was investigated by a Cox proportional hazards analysis using quartiles of homocysteine. Adjustment was made for age, sex, vitamin levels, creatinine, body mass index, smoking, and prevalence of hypertension, hyperlipidemia and diabetes at baseline. In the crude analysis, homocysteine was positively related to risk of myocardial infarction comparing participants of the highest quartile with those of the lowest (HR 2.0, 95% CI 1.2-3.4, *p* for trend 0.004). This association was only slightly altered after adjustment for age, sex and creatinine (HR 1.7, 95% CI 1.0-2.9, *p* for trend 0.041). However, after multivariate adjustment, the association between homocysteine and myocardial infarction was considerably attenuated. Subjects with the MTHFR 677TT genotype had higher homocysteine concentrations than those with the CT or CC genotype. The 677TT genotype was significantly more often present in cases than controls (17% versus 9%, *p*<0.04).

In conclusion, homocysteine appeared to be a risk factor for myocardial infarction in the prospective EPIC Potsdam study, however, it was not independent of other established risk factors.

C024

**HOMOCYSTEINE AND CHLAMYDIA PNEUMONIAE IN HEALTHY SUBJECTS AND IN PATIENTS WITH CORONARY ARTERY DISEASE**Stanger O,<sup>1</sup> Erwa W,<sup>2</sup> Strele A,<sup>3</sup> Pierich C,<sup>5</sup> Marz W,<sup>2</sup> Hufnagl C,<sup>1</sup> Tiran A<sup>4</sup>*<sup>1</sup>Cardiac Surgery, Cardiovascular Research, Private Medical University Salzburg, Austria; <sup>2</sup>Institute of Medical and Chemical Laboratory Diagnostics, <sup>3</sup>Biostatistics, <sup>3,4</sup>Center for Medical Research, all Medical University of Graz, Austria, <sup>5</sup>Nuclear Medicine/Endocrinology, Private Medical University Salzburg, Austria*

**Background.** Chlamydia pneumoniae (*C. pneumoniae*) depend on folate for replication. We previously described a strong relationship between elevated levels of homocysteine (tHcy) and infection with *C. pneumoniae* in patients with coronary artery disease (CAD). The atherogenic role of this interaction is unclear.

**Aims.** We investigated the potential contribution of tHcy

and *C. pneumoniae* infections to accelerated atherosclerosis.

**Methods.** 315 patients with CAD (52.4±5.5 years) were matched with 315 healthy controls for sex and age (±2mo). Chlamydial antibodies to a recombinant genus-specific lipopolysaccharide (LPS), Hcy, folate, vitamin B12, lipids and hsCRP were measured in all 630 participants.

**Results.** Seventy-six subjects were classified hyperhomocyst(e)inemic (fasting homocysteine >14 mmol/L), and 554 subjects were below cut-off (tHcy <14 mmol/L). Overall seropositivity was 43.3%. IgG titers (1.42±1.58 vs 1.35±1.2, *p*=0.24) were not significantly different between patients and controls. We found a strong correlation between seropositivity in hyperhomocysteinemia (64.6 vs. 37.4, *p*<0.001) and IgG antibody titers in patients (1.77±1.65 vs. 1.35±1.56, *p*=0.001), and with borderline significance also in healthy controls (1.85±1.59 vs. 1.31±1.26, *p*=0.056). Overall titers correlated significantly with tHcy levels in patients and controls. Subjects with Hcy > 14 μmol/l had significantly higher tHcy levels as compared with Hcy<14 μmol/L (10.74±3.97 vs. 9.92±2.92, *p*=0.014). HsCRP was mostly determined by HDL-cholesterol and only weakly by Hcy and IgG titers.

**Conclusions.** The strong correlation between homocysteine and infection with *Chlamydia pneumoniae* persists in atherosclerotic patients and in subjects free of vascular disease. Thus this association is likely to represent an intrinsic interaction between the microorganism and the host. Seropositivity is associated with higher plasma homocysteine in all, but does not identify CAD-patients from controls.

**WORKSHOP 4****HOMOCYSTEINE and RELATED BIOMARKERS - METHODS of ASSESSMENT**

C025

**BIOCHEMICAL INDEXES OF THE B-VITAMINS IN CORD SERUM ARE PREDICTED BY MATERNAL B-VITAMIN STATUS**Obeid R,<sup>1</sup> Munz W,<sup>2</sup> Herrmann W<sup>1</sup>*<sup>1</sup>Department of Clinical Chemistry; <sup>2</sup>Department of Obstetrics and Gynecology, University Hospital of Saarland, Homburg/Saar, Germany*

**Background.** Vitamin B12 is the major determinant of plasma concentrations of homocysteine (Hcy) in newborn infants. Maternal status of folate, vitamin B12 and B6 during pregnancy might impact biochemical markers of these micronutrients in the newborns.

**Aims.** We studied the relationship between concentrations of the metabolites and the B-vitamins in maternal sera and umbilical venous blood of their newborns.

**Methods.** We studied healthy pregnant women at labor who were expecting healthy- term- and appropriate-birth-weight babies. Samples were available from 120 mother-baby pairs (9 IUGR, 16 pre-term and 95 term babies with appropriate birth weight).

**Results.** Concentrations of the B-vitamins were higher in cord samples as compared to maternal blood (folate; 2-folds, B12; 1.5-folds and B6; 6-folds). Concentrations of cystathionine and methylmalonic acid (MMA) were also higher in the babies as compared to the mothers (mean (SD) cystathionine; 462 (189) vs. 343 (143) nmol/L and MMA; 353 (144) vs. 233 (110) nmol/L). Concentrations of holotranscobalamin (holoTC) were higher in the babies as com-

pared to the mothers (mean (SD)=119(74) vs. 55(29) pmol/L). No significant differences in Hcy concentrations were observed between fetal and maternal samples. Higher fetal-MMA was related to higher maternal MMA and B12 and lower fetal concentrations of B12. Fetal concentrations of cystathionine concentrations were determined by maternal cystathionine, gestational age, fetal B6 and Hcy. No significant differences in serum concentrations of Hcy or MMA were detected between term, pre-term or IUGR-babies. Values of the metabolites and the vitamins did not differ significantly between umbilical venous and arterial blood.

**Conclusions.** Maternal concentrations of metabolic markers of the B-vitamins predict values in fetal blood at delivery. Fetal homocysteine concentrations were low but were determined by the vitamin status of the mother. The effect of increasing maternal intake of vitamin B12 and B6 during pregnancy on the fetal concentrations of the metabolites should be investigated.

### C026

#### EFFECT OF WHEAT FLOUR FORTIFICATION WITH B VITAMIN COMPLEX AND IRON ON PLASMA HOMOCYSTEINE, FOLATE, VITAMIN B12 AND FERRITIN CONCENTRATIONS IN HEALTHY ISRAELI WOMEN

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**Background.** In recent years, folic acid, has come into focus due to its protective role against neural tube defects and possibly, cardiovascular disease and certain cancers. In light of this, the USA Food and Drug Administration has recommended, in 1998, that cereal-grain products be fortified with folic acid.

**Objective.** To investigate the effects of flour fortification used for bread with B-vitamin complex and iron on folate, vitamin B12, homocysteine and ferritin status, in order to establish a baseline for monitoring the fortification program in Israel.

**Design.** Single-blind, randomized placebo-controlled intervention trial.

**Setting.** Meir General Hospital, Kefar Sava, Israel.

**Subjects.** 246 women employee at Meir hospital (age 18-58 years)

**Duration.** Six weeks.

**Intervention.** Daily consumption based on the usual consumption, of unfortified bread (placebo) or bread fortified, per 100 g flour, with 140 mcg folic acid, 1 mcg vitamin B12, 0.58 mg thiamin, 0.39 mg riboflavin, 4.7 mg niacinamide and 4.4 mg iron. Food Frequency Questionnaires were used to assess the dietary intake. The consumption of other commercially fortified grain products were avoided.

**Preliminary results.** Mean plasma folate increased from 8.6 to 9.9 ng/mL in the treatment group vs 8.9 to 9.7 ng/mL in the placebo group ( $p=0.005$ ). For vitamin B12 the increase in concentrations were from 415 to 478 pg/mL in the treatment group vs 419 to 455 pg/mL in the placebo group ( $p=0.000$ ). There was no significant effect on mean tHcy concentrations. However among those with high tHcy ( $>11.3$   $\mu\text{mol/L}$ ) there was a decrease in the mean concentration from 12.1 to 10.4 in the treatment group vs 12.4 to 11.8  $\mu\text{mol/L}$  in the placebo group ( $p=0.005$ ). For plasma ferritin, there was no change in concentration among those with high levels. However among those with lower values ( $<16$  ng/mL) the mean plasma ferritin increased from 9.7 to 12.8 ng/mL in the treatment group vs a slight decrease (9.5 to 8.8 ng/mL) in the placebo group ( $p=0.037$ )

**Conclusions.** The fortification of wheat flour with B complex and iron was associated with a substantial improvement in folate, vitamin B12, homocysteine and ferritin status in a population of childbearing age women in Israel.

### C027

#### THE FRACTION OF SERUM VITAMIN B12 CIRCULATING AS HOLOTRANSCOBALAMIN IS INCREASED IN VITAMIN B12 DEFICIENT VEGAN MEN

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**Background.** Holotranscobalamin (holoTC) is considered the bioavailable form of cobalamin in serum representing approximately 25% of total serum vitamin B12 with the remaining 75% as holohaptocorrin. It has been suggested that in hyperhomocysteinaemic vegan populations, a lower proportion of cobalamin is bioavailable and that this may constitute a clinical risk not apparent from serum vitamin B12 levels.

**Aims.** To investigate the fraction of the circulating cobalamin present as holoTC, as a function of the total serum vitamin B12 concentration in a vegan population in which homocysteine (tHcy) levels were determined primarily by cobalamin with folate levels being high and other cardiovascular risk factors being absent.

**Methods.** Homocysteine and methylmalonic acid (MMA) were measured using isotope dilution GCMS, holoTC by ELISA, and serum vitamin B12 by a competitive protein binding immunoassay.

**Results.** Results are presented for a population of 161 vegan men, 86 of whom had serum vitamin B12 levels  $<200$  ng/L. The percent of serum vitamin B12 present as holoTC, has been distributed according to quantiles of serum vitamin B12 for the  $<200$  ng/L and  $>200$  ng/L sub-groups. Quintile regression analysis indicated a positive association ( $p=0.001$ ) between serum vitamin B12 and percent serum vitamin B12 as holoTC in the  $<200$  ng/L sub-group. In the lowest quantile of the  $<200$  ng/L sub-group, serum vitamin B12,  $31.4\pm 5.3$  (CI) ng/L (tHcy  $44.1$   $\mu\text{mol/L}$ , MMA  $1.1$   $\mu\text{mol/L}$ ), all serum vitamin B12 appeared to be as holoTC ( $111.0\pm 23.4$  (CI)% ng/L). In the lowest quantile of the  $>200$  ng/L sub-group, serum vitamin B12,  $234\pm 7.4$  (CI) ng/L (tHcy  $12.4$   $\mu\text{mol/L}$ , MMA  $0.30$   $\mu\text{mol/L}$ ), the fraction of serum vitamin B12 accounted for by holoTC,  $28.9\pm 3.9$  (CI)%, was significantly less ( $p<0.001$ ) than in the  $<200$  ng/L sub-group.

**Conclusions.** At serum vitamin B12 levels normally associated with clinical symptoms of deficiency a switch occurs, enabling the conservation of bioavailable cobalamin.

## C028

**RECOMBINANT HUMAN INTRINSIC FACTOR INCREASES THE VITAMIN B12 ABSORPTION AMONG INDIVIDUALS WITH OBVIOUS BIOCHEMICAL SIGNS OF VITAMIN B12 DEFICIENCY**Hvas AM,<sup>1</sup> Buhl T,<sup>2</sup> Laursen NB,<sup>3</sup> Hesse B,<sup>2</sup> Berglund L,<sup>3</sup> Nexø E<sup>1</sup><sup>1</sup>Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark; <sup>2</sup>Department of Nuclear Medicine & PET, Copenhagen University Hospital, Copenhagen, Denmark; <sup>3</sup>Protein Chemistry Laboratory, Department of Molecular Biology, Aarhus University, Aarhus, Denmark

We have developed a vitamin B12 absorption test "Cobasorb" based on measurement of an increase in plasma holotranscobalamin (holoTC) measured by ELISA and the use of recombinant human intrinsic factor (rhIF) produced in plants. We included 35 patients (15 males and 20 females, aged 22-84 (median 58) years) with plasma cobalamins below the lower reference limit (<200 pmol/L). We obtained blood samples on day 1, day 2 (24 hours after 3 x 9 microgram vitamin B12 given six hours apart) and day 3 (24 hours after intake 3 x 9 microgram vitamin B12 together with rhIF given six hours apart). HoloTC increased more than 15% and/or more than 15 pmol/L from day 1 to day 2 in 16 patients indicating an active uptake of free vitamin B12. Among the remaining 19 patients, 11 patients had a small increase in holoTC both from day 1 to 2 (mean increase; 8 pmol/L) and from day 2 to 3 (mean increase; 6 pmol/L) suggesting that these patients suffered from intestinal malabsorption. The remaining 8 patients had a significantly higher uptake between day 2 and 3 (mean increase; 16 pmol/L) than between day 1 and day 2 (mean increase; 4 pmol/L),  $p < 0.0001$ , suggesting lack of intrinsic factor, since rhIF was capable of restoring the absorption of vitamin B12. These 8 patients had obvious biochemical signs of vitamin B12 deficiency as compared to the remaining as judged from plasma cobalamins ( $p = 0.005$ ), plasma methylmalonic acid ( $p = 0.0001$ ) and plasma homocysteine ( $p = 0.12$ ). In conclusion we show that rhIF increases the vitamin B12 absorption among patients with obvious biochemical signs of vitamin B12 deficiency.

## C029

**DETERMINATION OF FOLATE IN FRESH AND STORED SERUM AS PARA-AMINOBENZOYLGLUTAMATE**

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**Background.** For serum folate, Electronic Quality Control and assay inter-laboratory calibration are difficult, mostly because folates are unstable. This makes production, transportation, storage and distribution of quality control specimens and samples impractical. Such instability also creates great problems when carrying out large epidemiological studies based on stored samples.

**Aims.** All the different folate species have common structural features which include a pteridine residue and para-aminobenzoylglutamate (pABG). The principle behind the pABG assay is to measure all folate species as pABG equivalents.

**Methods.** Folate in serum was oxidized (using potassium permanganate) and subjected to limited acid hydrolysis. Under these conditions different folate species were converted to pABG whereas essentially no para-aminobenzoic

acid (pABA) was formed. pABG was quantified by LC-MS/MS.

**Results.** The detection limit was 0.5 nmol/L in serum, i.e. far below the concentration of folate in serum. The pABG assay was verified by comparison of folate concentration in fresh serum samples as determined by the microbiological assay and the pABG assay. A strong correlation was observed (Pearson's  $r$  of 0.97). Also the folate concentration in serum samples stored at  $-20^{\circ}\text{C}$  for about 25 years were determined by both methods. In these samples, 8 out of 10 samples had folate concentration far below the normal reference limits as determined by the microbiological assay. In contrast, all samples had essentially normal folate concentration when determined by the pABG method.

**Conclusions.** The pABG assay correlates well with the microbiological assay in fresh samples. In stored samples, the pABG assay seems to measure folate species that are degraded, and therefore not detectable by conventional folate assays.

## C030

**INFLUENCE OF TC2 I23V POLYMORPHISM ON HOMOCYSTEINE AND SAM LEVELS IN PREGNANT WOMEN**Trentin R,<sup>1</sup> Stabler SP,<sup>2</sup> Hirata MH,<sup>1</sup> Hirata RDC,<sup>1</sup> Allen RH,<sup>2</sup> Sampaio-Neto LF,<sup>3</sup> Guerra-Shinohara EM<sup>1</sup><sup>1</sup>University of Sao Paulo, Brazil; <sup>2</sup>University of Colorado Health Science Center, USA; <sup>3</sup>Pontificia Universidade Catolica de Sao Paulo, Brazil

Cobalamin attached to transcobalamin II is the active cobalamin fraction taken up by tissues. Polymorphisms in the gene of transcobalamin II (TC2) could affect cobalamin concentrations in tissues and consequently could elevate homocysteine (tHcy) levels. We investigated the effects of TC2 I23V polymorphism on homocysteine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels and the effects of association between lower cobalamin levels and TC2 I23V genotypes. Genotypes for this polymorphism were determined by PCR-FLRP. The levels of cobalamin, red blood cell folate, serum folate, tHcy, SAM, SAH and SAM/SAH ratio were determined in 369 pregnant women (37-42 weeks of gestational age). Pregnant women with 23V allele had lower SAM levels than did women with 23I allele. Low levels of cobalamin are associated with high risk for increased tHcy in pregnant women independently of TC2 I23V genotypes (Table). We concluded that TC2 23V allele can be associated with changes on SAM levels and it may be contribute to increased of risk for elevated tHcy levels in pregnant women.

**Table. Interaction between TC2 I23V polymorphism and cobalamin levels in pregnant women.**

Dependent variables	Independent variables (N)	P-value	O.R.	95% CI
	II genotype and cobalamin >180,8 pmol/L (61)	----	1.00 (ref)	----
tHcy >8.3 $\mu\text{mol/L}$ (363)	II genotype and cobalamin "180,8 pmol/L (196)	0.015	2.86	1.22-6.68
	IV+VV genotypes and cobalamin >180,8 pmol/L (31)	0.310	1.85	0.56-6.08
$\leq$	IV+VV genotypes and cobalamin "180,8 pmol/L (75)	0.010	3.41	1.35-8.63

*P for trend:  $p = 0.027$ ; the cobalamin and tHcy cutoff values were the highest quartiles.*

## C031

**VITAMIN B6 FORMS AND THEIR RELATIONSHIP TO HOMOCYSTEINE, CYSTATHIONINE AND CYSTEINE IN HUMAN PLASMA**Midttun O,<sup>1</sup> Hustad S,<sup>1</sup> Schneede J,<sup>1</sup> Grotmol T,<sup>2</sup> Ueland PM<sup>1</sup><sup>1</sup>LOCUS for Homocysteine and Related Vitamins, University of Bergen; <sup>2</sup>The Cancer Registry, Oslo, Norway

**Background.** Three major forms of vitamin B6 exist in human plasma, pyridoxal phosphate (PLP), pyridoxal (PL) and the catabolite pyridoxic acid (PA). Vitamin B6 status is frequently assessed by determining plasma concentrations of PLP, but other B6 vitamers have also been used.

**Aims.** To investigate the distribution of the three major vitamin B6 forms in human plasma, and investigate their relationship to plasma total homocysteine (tHcy), cystathionine and total cysteine.

**Methods.** 2546 men and women aged 50-65 years were selected from the NORCCAP (Norwegian Colorectal Cancer Prevention) cohort (n=10700) according to MTHFR genotype and equally distributed between the CC, CT and TT genotypes. Blood samples were analysed by LC-MS/MS (tHcy, cystathionine, PLP, PL and PA) and GC-MS (total cysteine). Data were analysed by Spearman correlations and multiple linear regression.

**Results.** Median (5th, 95th percentiles) concentrations were 10.2 nanomol/L (6.5, 19.0) for Hcy, 0.183  $\mu$ mol/L (0.083, 0.518) for cystathionine, 278 micromol/L (226, 334) for cysteine, 43.7 nanomol/L (15.7, 138.5) for PLP, 9.5 nanomol/L (5.0, 38.5) for PL and 20.3 nanomol/L (10.0, 100.3) for PA. Spearman coefficients for the correlation between the vitamin B6 species were 0.81 (PLP vs. PL), 0.69 (PLP vs. PA) and 0.80 (PL vs. PA) ( $p < 0.001$ ). The associations between the vitamin B6 forms and tHcy were strongest in subjects with the TT genotype. In the TT group, the estimated tHcy difference between subjects with concentrations of plasma PLP in the lowest compared to the highest quartile was 4.1  $\mu$ mol/L in a multiple linear regression model which was adjusted for age, sex, folate, cobalamin and creatinine. The corresponding tHcy difference was 3.7  $\mu$ mol/L for PL and 3.3  $\mu$ mol/L for PA. For cystathionine and cysteine, associations with the B6 forms were weak or absent.

**Conclusions.** There was a strong correlation between concentrations of the three major vitamin B6 forms, PLP, PL and PA. All B6 forms were related to plasma tHcy in a multivariate model, but only weakly related to cystathionine and total cysteine.

## C032

**S-ADENOSYLMETHIONINE AND S-ADENOSYLMETHIONINE DETERMINATION BY STABLE-ISOTOPE DILUTION LC-ESI-MS/MS**Gellekink H,<sup>1,2</sup> Van Oppenraaij-Emmerzaal D,<sup>1</sup> Van Rooij A,<sup>1</sup> Struys EA,<sup>4</sup> Den Heijer M,<sup>2,3</sup> Blom HJ<sup>1</sup><sup>1</sup>Laboratory of Pediatrics and Neurology; <sup>2</sup>Department of Endocrinology; <sup>3</sup>Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; <sup>4</sup>Metabolic Unit, Department of Clinical Chemistry, VU University Medical Center Amsterdam, Amsterdam, The Netherlands

**Background.** It is thought that a change in S-adenosylhomocysteine provides a mechanism for homocysteine-related pathology.

**Aims.** We developed a method to measure S-adenosylhomocysteine and S-adenosylmethionine in plasma by stable-isotope dilution LC-MS/MS. Acetic acid was added to pre-

vent S-adenosylmethionine degradation.

**Methods.** Solid-phase extraction columns were used to bind S-adenosylmethionine, S-adenosylhomocysteine, their internal standards and for sample cleanup. Separation and detection was achieved by coupling a chromatographic column directly to the LC-MS/MS.

**Results.** The method was linear over a range of 10 to 800 nmol/L for S-adenosylmethionine and 5 to 400 nmol/L for S-adenosylhomocysteine ( $r > 0.999$ ). The interassay CV for S-adenosylmethionine and S-adenosylhomocysteine were 3.9% and 8.3%, respectively, and the intraassay CV were 4.2% and 6.7%, respectively. Mean recovery for S-adenosylmethionine was 94.5% and 96.8% for S-adenosylhomocysteine and quantification limits were 2.0 and 1.0 nmol/L for S-adenosylmethionine and S-adenosylhomocysteine, respectively. S-adenosylmethionine appeared unstable but immediate sample acidification with acetic acid prevented degradation. In a group of controls (mean tHcy 11.2 mmol/L), mean plasma S-adenosylmethionine and S-adenosylhomocysteine were 94.5 nmol/L and 12.3 nmol/L, respectively.

**Conclusions.** Stable-isotope dilution LC-MS/MS allows a rapid determination of S-adenosylmethionine and S-adenosylhomocysteine. No metabolite derivatization is needed and sample preparation time is minimal. The instability of S-adenosylmethionine is a serious problem and can be prevented by direct acidification of the samples.

## WORKSHOP 5

**REPRODUCTION and EARLY DEVELOPMENT**

## C033

**A TRANSIENT REDUCTION IN tHcy IN PREGNANCY IS REVERSED AT DELIVERY AND AFTER 6 YEARS POST PARTUM, WITH THE LATTER ALSO PREDICTING VALUES IN THE CHILDREN**Murphy MM,<sup>1</sup> Fernandez-Ballart JD,<sup>1</sup> Arijia V,<sup>1</sup> Molloy AM,<sup>2</sup> Scott JM<sup>2</sup><sup>1</sup>Area of Preventive Medicine & Public Health, Faculty of Medicine & Health Sciences, Universitat Rovira i Virgili, Reus, Spain; <sup>2</sup>Department of Biochemistry, Trinity College, Dublin, Ireland

**Background.** A physiological reduction in tHcy occurs during pregnancy which is enhanced by folic acid supplementation.

**Aims.** To investigate 1) whether tHcy has returned to baseline 6 years post-partum regardless of whether mothers used folic acid supplements during pregnancy; 2) whether maternal tHcy before pregnancy, at labour and 6 years post partum predicts the child's tHcy.

**Methods.** Plasma tHcy was determined in samples collected from 92 women 2-10 weeks preconception, at 8, 20 and 32 weeks of pregnancy, during early labour, from the umbilical cord and 6 years later from 75 mothers and their 6-year old children.

**Results.** The reduction in tHcy (micromol/L, geometric mean (SD) from preconception: 8.14 (1.28) to 6.49 (1.30), 5.21 (1.30), 5.16 (1.32) at 8, 20 and 32 weeks respectively of pregnancy, was reversed by labour 7.92 (1.39) in women unsupplemented with folic acid but was significantly lower than baseline 8.33 (1.36) in women supplemented (39) during the 2nd / 3rd trimester 6.23 (1.48),  $p < 0.0001$  ANOVA. Six years post partum tHcy had returned to baseline in unsupplemented 8.00 (1.31) and supplemented 8.58 (1.28) women. tHcy at preconception was significantly correlated with tHcy at labour, 6 years post partum, cord tHcy and tHcy in the 6

year old child in unsupplemented women ( $r: 0.4, p < 0.01$ ;  $r: 0.7, p < 0.05$ ;  $r: 0.4, p < 0.05$ ;  $r: 0.3, p < 0.05$  respectively) and with labour and 6 years post partum in supplemented women ( $r: 0.7, p < 0.01$ ;  $r: 0.7, p < 0.01$ ). There was a significant correlation between mother and child tHcy 6 years later (unsupplemented  $r: 0.3, p < 0.05$ ; supplemented  $r: 0.6, p < 0.01$ ).

**Conclusions.** Despite exposure to variables which affect tHcy such as pregnancy folic acid supplementation and other lifestyle confounders, baseline tHcy measurements more than 6 years apart were similar and there was an association between tHcy in mothers and their children. This provides evidence for stability of tHcy with time and for the validity of once off tHcy determinations.

**Table. Geometric mean tHcy (SD)(mmol/L). [n]**

	Weeks of pregnancy					Child		
	Preconception	8	20	32	Labour	6 years post partum	Cord	6 years
<b>US</b>	<b>8.14</b> (1.28) [52]	6.49 <sup>a</sup> (1.30) [53]	5.21 <sup>a</sup> (1.30) [52]	5.16 <sup>a</sup> (1.32) [52]	<b>7.92</b> <sup>□</sup> (1.39) [46]	8.00 <sup>□</sup> (1.31) [43]	6.36 <sup>□□</sup> (1.45) [46]	5.87 <sup>□□</sup> (1.30) [43]
<b>S</b>	<b>8.33</b> (1.36) [36]	6.36 <sup>a</sup> (1.35) [34]	4.18 <sup>ab</sup> (1.32) [37]	4.44 <sup>ac</sup> (1.36) [37]	<b>6.23</b> <sup>ad</sup> <sup>□</sup> (1.48) [36]	8.58 <sup>□</sup> (1.39) [33]	5.21 <sup>d</sup> <sup>□</sup> (1.28) [35]	5.87 <sup>□□</sup> (1.19) [32]

(US: unsupplemented; S: supplemented with folic acid during the 2<sup>nd</sup> / 3<sup>rd</sup> trimester of pregnancy). <sup>a</sup>Lower than at preconception ( $p < 0.0001$ ) ANOVA repeated measures. Lower than unsupplemented\* ( $p < 0.0001$ ), <sup>b</sup>( $p < 0.05$ ), <sup>c</sup>( $p < 0.001$ ), Student's unpaired t-test. Correlations: with preconception <sup>□</sup>  $p < 0.01$ ; <sup>□□</sup>  $p < 0.05$ ; with labour <sup>□□</sup>  $p < 0.01$ ; <sup>□</sup>  $p < 0.05$ ; with mother 6 years post partum <sup>□□</sup>  $p < 0.05$ ; <sup>□</sup>  $p < 0.01$ .

### C034

#### THE MTHFD1 R653Q POLYMORPHISM AND COMPLICATIONS OF PREGNANCY

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**Background.** The MTHFD1 gene encodes the NADP-dependent cytoplasmic enzyme that encompasses three activities: 5,10-methylenetetrahydrofolate dehydrogenase, 5,10 methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase. Thus, MTHFD1 plays a central role in folate metabolism and is an important provider of the donor cofactors for purine/pyrimidine synthesis. An alteration in the supply of cofactors for DNA synthesis is likely to affect a range of developmental processes. Maternal folate status and/or homocysteine levels have been implicated in a range of pregnancy complications, most notably in pregnancies affected by a neural tube defect (NTD). We have previously identified the R653Q polymorphism in MTHFD1 as a maternal risk factor for NTDs. Specifically, women who are homozygous for the 'Q' allele (i.e., 'QQ') have an increased risk of an NTD-affected pregnancy (OR 1.52 (95% CI 1.16-1.99),  $p = 0.003$ ).

**Aims.** The aim of our study was to initially confirm the

association of the MTHFD1 R653Q polymorphism as a maternal risk factor for NTDs in the Irish population and also to consider whether the MTHFD1 R653Q polymorphism had a role to play in other pregnancy complications. **Methods.** We retrospectively identified women with a number of pregnancy complications from a bank of 56,049 blood samples drawn during their first clinical visit to one of the three Dublin maternity hospitals between 1986 and 1990. Control samples were a systematic random sample from the same bank.

**Results.** We have confirmed the association between the MTHFD1 'QQ' genotype and maternal NTD risk in a second cohort of women from the Irish population (OR 1.49 (95% CI 1.07-2.09),  $p = 0.02$ ). We also found that the MTHFD1 'QQ' genotype is a significant risk factor for severe abruptio placentae (OR 2.85 (95% CI 1.47-5.53),  $p = 0.002$ ) but not for pre-eclampsia (OR 1.44 (95% CI 0.78-2.67)  $p = 0.25$ ).

**Conclusions.** MTHFD1 R653Q is an important polymorphism to consider during pregnancy.

### C035

#### NOS3 GENETIC VARIANTS INTERACT WITH MATERNAL SMOKING AND MULTIVITAMIN USE DURING PREGNANCY TO ALTER RISK OF HUMAN OROFACIAL CLEFTS

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Brown and colleagues ('03) recently demonstrated that the endothelial nitric oxide synthase (NOS3) 894 polymorphism is associated with increased plasma homocysteine. Cigarette smoking also compromises NOS3 activity. Because risk of clefting has been independently associated with either maternal cigarette smoking or lack of folic acid supplementation (which may result in higher plasma homocysteine), we reasoned that genetic variation in NOS3 might interact with these two maternal exposures to influence clefting risks. Therefore, we genotyped 244 infants with isolated cleft lip (CLP), 99 infants with isolated cleft palate, and 588 controls from a large population-based California case-control interview study (1987-89 birth cohort) for three NOS3 polymorphisms: A(-922)G, C(-690)T, and G894T. Analyses of gene-only effects for each NOS3 SNP revealed a 60% increased risk for CLP among NOS3 922G homozygotes. We found some evidence for higher risk of CLP in infants whose mothers smoked cigarettes periconceptionally and who had a NOS3-922G allele (OR=2.5), but not for the 894T allele. We also found higher risks (odds ratios > 4) of CLP among mothers who smoked cigarettes, who did not use vitamins periconceptionally, and whose infants had at least one variant allele for each of these two NOS3 SNPs. No similar patterns were observed for risk of cleft palate. Risk estimates were not substantially different after adjusting for potentially confounding effects of maternal race/ethnic background. Infants homozygous for MTHFR 677T, whose mothers smoked and did not use vitamins periconceptionally, had odds ratio of 11.7 (1.2-118.6) if they had a NOS3-922G allele, and 7.2(0.6-82.4) if they had a NOS3 894T allele. Our population-based study of California infants suggests sizable increased risks of cleft lip from combined infant NOS3 genetic variation and maternal smoking. These risks may be further increased by MTHFR 677 genotype and lack of maternal vitamin use.

## C036

**MATERNAL MTHFR 677C>T COMBINED WITH INADQUATE PERICONCEPTIONAL FOLATE SUPPLEMENTATION; A RISK FACTOR FOR CONGENITAL HEART DEFECTS**

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In the etiology of congenital heart defects (CHD) both genetic and environmental factors are involved. Neural crest cells are essential for normal development of the heart. Ablation of the neural crest in chick embryos leads chiefly to conotruncal heart defects. Because of the involvement of neural crest cells it can be hypothesized that the pathogenesis of CHD is similar to pathophysiological mechanism of neural tube defects (NTD). The 677C>T polymorphism in the methylenetetrahydrofolate reductase (MTHFR)-gene is a genetic risk factor for NTD and possibly for CHD.

We investigated MTHFR 677C>T polymorphism as a genetic risk factor for CHD by conducting a case-control comparison and a transmission disequilibrium test in a family-based design. The effect of MTHFR 677C>T in combination with maternal periconceptional folate supplementation on CHD risk was also studied. A total number of 165 Caucasian families with nonsyndromic CHD-affected children participated in this study. The control group consisted of 261 women. The family-based transmission disequilibrium test analysis revealed no association of the fetal MTHFR 677TT genotype or 677T allele with the development of CHD in the embryo. In a case-only study the interaction between periconceptional folate supplementation with maternal MTHFR genotype was significant ( $p=0.012$ ). Less than 50% of the women were using periconceptional folate supplementation adequately. Maternal MTHFR 677CT and TT genotypes in combination with no periconceptional folate use was associated with respectively, a three-fold (OR 2.9 95%CI 1.35-6.41) and five-fold (OR 5.1 95%CI 1.88-13.99) increased risk for conotruncal heart defects in offspring. No risk was observed in women who used adequate periconceptional folate.

Our observation supports the hypothesis that an impaired folate and/or homocysteine metabolism interferes with the developing heart affecting the neural crest cells. Considering the genetic risk of maternal MTHFR 677CT and TT genotypes a substantial proportion of CHD might be prevented by adequate periconceptional folate supplementation.

## C037

**CHOLINE IS A DETERMINANT OF PLASMA HOMOCYSTEINE IN MATERNAL AND FETAL PLASMA**

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**Background.** Hepatic homocysteine metabolism is a major determinant of plasma total homocysteine (tHcy) levels. In liver, the oxidation of choline to betaine generates an important folate independent source of methyl groups for homo-

cysteine remethylation, thereby contributing to the maintenance of low plasma tHcy. During pregnancy, the high choline requirement for fetal growth and development places substantial stress on maternal hepatic choline stores. Thus, pregnancy may alter the balance of homocysteine metabolism between folate and choline dependent pathways.

**Aims.** The aim was to quantify the products of the choline oxidation pathway and assess the importance of this pathway compared with folate related metabolites as determinants of maternal and neonatal tHcy.

**Methods.** Blood was sampled from 201 pregnant women and from the umbilical cord veins at delivery of their healthy full term infants, and analysed for plasma free choline, betaine, dimethylglycine, folate, vitamin B12, tHcy and creatinine.

**Results.** Choline levels in neonatal plasma were three times higher than maternal plasma (median of 34.8 versus 12.2  $\mu\text{mol/L}$ ;  $p<0.0001$ ). Betaine and dimethylglycine were also significantly higher in neonates. In maternal blood, choline was positively associated with tHcy ( $r=0.34$ ;  $p<0.0001$ ), betaine ( $r=-0.58$ ;  $p<0.0001$ ) and dimethylglycine ( $r=0.30$ ;  $p<0.0001$ ). In a multiple regression model, adjusted for maternal creatinine, choline was a strong positive predictor of maternal plasma tHcy ( $p<0.0001$ ), whereas folate ( $p=0.06$ ), vitamin B12 ( $p=0.002$ ) and betaine ( $p=0.001$ ) were negative determinants. Much weaker relationships were seen in the neonatal circulation where maternal tHcy ( $p<0.0001$ ), neonatal vitamin B12 ( $p=0.008$ ), maternal vitamin B12 ( $p=0.018$ ) and maternal choline ( $p=0.009$ ) were significant determinants.

**Conclusions.** Elevated tHcy during pregnancy may be due to maternal de novo synthesis of choline induced when fetal demand exceeds dietary intake. Optimal dietary choline intake during pregnancy may help prevent elevated maternal tHcy in addition to ensuring adequate fetal and neonatal choline status.

## C038

**HOMOCYSTEINE, FOLATE, VITAMIN B12 AND METHYLMALONIC ACID LEVELS IN SPINA BIFIDA PATIENTS**

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Data show that folate levels in mothers with neural tube defect (NTD) offspring are towards the lower end of the reference range. Furthermore, elevated plasma and amniotic fluid homocysteine levels have been reported in mothers with a NTD affected child. Little is known about these metabolites in NTD patients. In the present study, we examined total homocysteine (tHcy), folate, vitamin B12 and methylmalonic acid (MMA) levels in plasma of 109 SB patients and 234 paediatric controls. Geometric mean plasma tHcy levels in SB patients (mean: 9.31  $\mu\text{mol/L}$ , 95%CI: 8.62-10.05) were 47.6% (35.9% to 60.3%) higher than those in the paediatric controls (mean: 6.24  $\mu\text{mol/L}$ , 95%CI: 5.93-6.56). Geometric mean plasma folate levels in SB patients (mean: 11.57  $\text{nmol/L}$ , 95%CI: 10.50-12.76) were 40.5% (47.0% to 33.4%) lower than those in the control group (mean: 19.14  $\text{nmol/L}$ , 95%CI: 17.90-20.49). SB patients had 20.9% (28.9%-11.9%) lower geometric mean plasma vitamin B12 levels (mean: 282  $\text{pmol/L}$ , 95%CI:

255-311) compared to the controls (mean: 349 pmol/L, 95%CI: 326-374). Mean plasma MMA levels did not differ between SB patients and paediatric controls (mean: 0.18 micromol/L, 95%CI: 0.16-0.19). Plasma tHcy levels above the 50th, 60th, 70th, 80th and 90th percentile were associated with a 1.8 to 3.6 fold increase in SB risk. Folate concentrations below the 50th, 40th, 30th, 20th and 10th percentile, increased SB risk from 3.4 to 6.9 fold. Both plasma vitamin B12 and plasma MMA levels did not increase the risk of SB. These results indicate that besides the maternal folate-homocysteine metabolism, disturbance of the fetal folate-homocysteine metabolism is a possible additional risk factor for SB.

### C039

#### THE METHIONINE SYNTHASE REDUCTASE 66A>G POLYMORPHISM IS A MATERNAL RISK FACTOR FOR SPINA BIFIDA

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The methionine synthase reductase (MTRR) enzyme restores methionine synthase (MTR) enzyme activity and therefore plays an essential role in the folate and vitamin B12-dependent remethylation of homocysteine to methionine. The 66A>G variant in the MTRR gene has been associated with neural tube defect (NTD) risk, however results are inconclusive. In this study, we examined the influence of the MTRR 66A>G polymorphism on spina bifida (SB) risk and studied the possible interaction between this variant and the MTR 2756A>G polymorphism, the MTHFR 677C>T polymorphism, plasma vitamin B12 levels and plasma methylmalonic acid (MMA) levels, in 121 mothers of a SB affected child, 109 SB patients, 292 control women and 234 paediatric controls. In our study population, the MTRR 66A>G variant had no significant effect on SB risk in SB patients themselves and rather exerted a protective effect (OR: 0.6; 95%CI: 0.4-1.1). In mothers, the MTRR 66GG genotype increased SB risk 2.1 fold (OR: 2.1; 95%CI: 1.3-3.3). This risk became even more pronounced in combination with the MTR 2756GG/AG genotype (OR: 3.0; 95%CI: 1.5-6.0) or the MTHFR 677TT genotype (OR: 4.0; 95%CI: 1.3-12.5). Moreover, we demonstrated an association between the MTRR 66GG genotype and high MMA levels, increasing maternal SB risk more than 5 times (OR: 5.5; 95%CI: 2.2-13.5). These data indicate that the MTRR 66GG genotype is a maternal SB risk factor, especially when intracellular vitamin B12 levels are decreased.

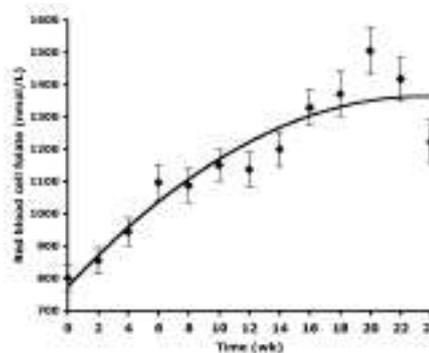
### C040

#### A SIX MONTH TIME COURSE OF RED BLOOD CELL FOLATE CONCENTRATIONS IN RESPONSE TO FOLIC ACID SUPPLEMENTATION

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Health authorities in several countries recommend that women planning a pregnancy take a daily supplement containing 400 µg folic acid from before conception until the 12th week of pregnancy to reduce the risk of their having a baby with a neural tube defect (NTD). It has not been established how long folic acid should be taken before conception to maximally reduce the risk, but NTD risk is inversely associated with maternal red blood cell (RBC) folate status. Using a microbiological assay, we measured the RBC folate con-

centrations of 24 young women following daily supplementation with 400 µg folic acid over 6 months in blood samples drawn every 2-weeks. The maximum mean increase in RBC folate concentration occurred at 20 weeks, increasing from a mean (SE) baseline value of 801 (41.1) to 1504 (73.3) nmol/L. The maximum increase was reached by 20 weeks in all but 2 participants. To obtain an estimate of the steady state mean RBC folate concentrations attained by the women consuming 400 µg/d of folic acid, we averaged the RBC folate values for weeks 20, 22, and 24. The mean (95% CI) increase above baseline values was 561 (461, 661) nmol/L. Half of this increase had been achieved by week 8. RBC folate concentrations in excess of 905 nmol/L have been associated with low risk of NTD; all women had exceeded this concentration by 16 weeks. Our data suggest that women planning a pregnancy should take a daily supplement containing 400 µg folic acid for a period of at least 16 weeks before conception to achieve the maximum protection afforded against a NTD-affected pregnancy. Further, supplementation trials in which the effect of folic acid on RBC folate concentrations is assessed, would need to be of at least 20 weeks duration to observe the maximum increase.



### WORKSHOP 6

#### BIOCHEMISTRY - PHYSIOLOGY - GENETICS - DNA METHYLATION

### C041

#### AN ACUTE DECREASE IN PLASMA TOTAL HOMOCYSTEINE PROMOTED SPECIFICALLY BY A SINGLE DOSE OF 5-METHYL-6S-TETRAHYDROFOLATE

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Numerous studies have examined the effect of many weeks of folate, mostly as folic acid, on lowering plasma total homocysteine (t-Hcy). At least one report suggests that a week is required for full response. We examined acute changes in t-Hcy following a single dose of folic acid (0, 0.4, 1.0, 2.5, or 5.0 mg), or 5-methyl-6S-tetrahydrofolate (5-MTHF; 1.04 or 5.2 mg). Subjects were healthy, and not consumers of supplements or appreciable fortified cereals, and

fasted for at least eight hours. Nine blood samples were taken over 6.5 h and a tenth 24 h (again fasting) after the dose. The average t-Hcy concentration decreased by ~6% in placebo subjects, with most of the drop occurring within 1.5 h. A similar, but less pronounced, decrease in plasma albumin was observed, suggesting both changes were due to an increase in circulating blood volume associated with recumbency. As previously reported, there was no difference from placebo in the t-Hcy curve with any dose of folic acid. However, with subjects having baseline t-Hcy > 6.8 microM (the median) the 5.2 mg dose of 5-MTHF resulted in a significant ( $p < 0.002$ ) decrease in t-Hcy of 14% by 5 h, three hrs after Cmax for plasma 5-MTHF. By 24 h t-Hcy returned to within 3% of baseline. The t-Hcy profile in subjects with baseline <6.8 microM was not different from placebo regardless of treatment. The acute lowering of t-Hcy in subjects with higher baseline levels is likely related to use of a large dose (relative to the total body pool) of immediately active folate, 5-MTHF. The observation that plasma t-Hcy, which is primarily protein-bound, can be altered so quickly has considerable implications for the mechanism of its regulation and transport. Supported by grants from NIH HL68165 and PamLab, LLB.

### C042

#### TRANSLATIONAL CONTROL OF SERINE HYDROXYMETHYLTRANSFERASE EXPRESSION THROUGH A FERRITIN-RESPONSIVE INTERNAL RIBOSOME ENTRY SEQUENCE

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Expression of the heavy chain ferritin (HCF) cDNA in cell culture models increases cytoplasmic serine hydroxymethyltransferase (cSHMT) protein levels and accelerates de novo dTMP synthesis while impairing homocysteine remethylation without altering cSHMT mRNA levels (J. Biol. Chem. 276, 19855-19861). The effect of HCF on cSHMT translation rates was determined by *in vitro* translation and mRNA transfection of bicistronic mRNA templates. The results demonstrate that the human cSHMT 5' untranslated region (5'-UTR) contains a HCF-responsive internal ribosome entry sequence (IRES); IRES activity is depressed in HCF-deficient mouse embryonic fibroblasts but stimulated in MCF-7 cells with increased HCF expression. Yeast two hybrid studies indicate that HCF interacts with the mRNA binding protein Bruno-L2; the cSHMT 3'UTR contains a consensus Bruno cis element and the 5'IRES is shown to be stimulated by the presence of the cSHMT 3'UTR in *in vitro* translation assay. This discovery represents a novel mechanism for translational regulation in eukaryotes. Furthermore, the murine cSHMT 5UTR is not a functional IRES and this observation may account for explain the inability to model the physiological effects of human iron deficiency on folate metabolism in rodents.

### C043

#### METABOLIC EFFECTS OF POLYMORPHISMS IN GENES RELATED TO THE FOLATE AND HOMOCYSTEINE METABOLISM IN A NORWEGIAN POPULATION

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**Background.** The methylenetetrahydrofolate reductase (MTHFR) 677C>T is an important genetic determinant of

folate and homocysteine status and has significant disease associations. The role of other polymorphisms related to one-carbon metabolism is still debated, and large study populations may be required to obtain sufficient statistical power to ascertain their metabolic effects.

**Aims.** Determination of prevalence of 13 different polymorphisms and their influence on folate/homocysteine metabolism in a Norwegian population.

**Methods.** About 10.000 subjects recruited to the Norwegian Colorectal Cancer Prevention study (NORCCAP) of an average-risk Norwegian population 50-64 of age were screened by a high-throughput assay based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). The assay includes 13 different polymorphisms related to folate and homocysteine metabolism: methylenetetrahydrofolate reductase (MTHFR) 677C>T and 1298A>C, methionine synthase (MTR) 2756A>G, methionine synthase reductase (MTRR) 66A>G, cystathionine beta-synthase (CBS) 844ins68 and 699C>T, transcobalamin-II (TC-II) 776C>G and 67A>G, reduced folate carrier-1 (RFC1) 80G>A, paraoxonase-1 (PON1) 575A>G and 163T>A, betaine homocysteine methyltransferase (BHMT) 742G>A, and methylenetetrahydrofolate dehydrogenase (MTHFD1) 1958G>A. Genotypes were studied in relation to concentrations of total homocysteine (tHcy), cobalamin, methylmalonic acid, methionine, creatinine, cysteine, choline, betaine, dimethylglycine, serum plasma folate, and different forms of vitamin B2 and B6.

**Results.** Allele frequencies were 0.28 (MTHFR 677T), 0.34 (MTHFR 1298C), 0.19 (MTR 2756G), 0.60 (MTRR 66G), 0.09 (CBS 844ins68), 0.33 (CBS 699T), 0.44 (TCII 776G), 0.12 (TCII 67G), 0.42 (RFC1 80A), 0.28 (PON1 575G), 0.33 (PON1 163A), 0.29 (BHMT 742A), and 0.45 (MTHFD1 1958A). As expected, tHcy level was increased and plasma folate was decreased according to the number of MTHFR 677T alleles. Significant increase of tHcy and decrease of plasma folate were also observed for the MTHFR 1298CC within the MTHFR 677CC subgroup. The MTR 2756A>G was significantly associated with tHcy level. Modest effects on various metabolites were also observed for the TC-II 776C>G, BHMT 742G>A, and MTHFD1 1958G>A polymorphisms.

**Conclusions.** Measuring a panel of metabolites and genotyping in a large population of 10000 subjects confirmed strong effect of polymorphisms related to one-carbon metabolism, but also allowed the unequivocal assessment of weaker and combined effects.

### C044

#### INTERACTION BETWEEN FOLATE AND MTHFR 1298 A>C POLYMORPHISM ON GENOMIC DNA METHYLATION

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**Background.** Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyl tetrahydrofolate, which serves as a methyl donor in the conversion of homocysteine to methionine, and thereby reduced MTHFR activity results in elevation of total plasma homocysteine. Epidemiologic studies have shown that two functional polymorphisms in MTHFR gene, 677C>T and 1298A>C, are related to increased cancer risk.

**Aims.** To determine whether the interaction between folate and MTHFR 1298A>C polymorphism affects genomic DNA methylation as a candidate mechanism for the relationship between this polymorphism and cancer risk.

**Methods.** From 198 unrelated, age- and sex-matched subjects genomic DNA methylation in lymphocytes was measured using liquid chromatography/mass spectrometry. Each genotype of MTHFR polymorphisms was determined by PCR and restriction fragment length analysis. Plasma folate and total homocysteine concentrations were measured by an automated chemiluminescence method and HPLC method.

**Results.** Subjects with 1298AA wild-type showed lower genomic DNA methylation compared to those with 1298AC or 1298CC genotypes (3.72 vs 8.59 or 6.79 ng 5-mCyt/μg DNA,  $p < 0.0001$  and  $p = 0.007$ , respectively). When DNA methylation was evaluated according to plasma folate status, only 1298AA subjects with low folate levels revealed diminished DNA methylation ( $p < 0.0001$ ). Moreover, when two MTHFR polymorphisms were concomitantly evaluated at the low folate status, DNA methylation was reduced only in 1298AA/677TT compared to 1298AA/677CC (3.11 vs 7.29 ng 5-mCyt/μg DNA,  $p = 0.001$ ) and to 1298CC/677CC genotypes (3.11 vs 7.14 ng 5-mCyt/μg DNA,  $p = 0.004$ ). However, the high prevalence of 677TT mutants within the 1298AA group (79%) and the similar molecular and biochemical features of 1298AA/677CC and 1298CC/677CC combined genotypes suggest that the gene-nutrient interaction affecting DNA methylation in 1298AA is mainly due to the coexistence of the 677TT genotype.

**Conclusions.** The 1298A>C polymorphism may convey its protective effect not through gene-nutrient interaction affecting DNA methylation in 1298AA, but through other pathway in one-carbon metabolism.

#### C045

##### ELUCIDATION OF FOLATE MEDIATED CASCADES IN THE DEVELOPING NEURAL TUBE: <BR> CONGENITAL MALFORMATIONS SECONDARY TO METHYLTRANSFERASE INHIBITION

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Folic acid is involved in several biochemical pathways including nucleoside biosynthesis and the production of methyl groups via the methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and homocysteine pathway. Supplementation with folic acid has been shown to have beneficial effects, which include decreasing homocysteine levels and reducing the risk of neural tube defects (NTDs). In order to study the interactions of folic acid with homocysteine levels, and the mechanism by which folic acid reduces the occurrence of congenital malformation, we utilized folate binding protein 1 (STOCK-Folbp1tm1Fnn) mice. Folbp1 is involved in maternal-to-fetal transport of folate, and the Folbp1<sup>-/-</sup> mice have been previously shown to present with congenital malformations, specifically NTDs. Rescuing of Folbp1 KO mice from lethality and NTDs was performed by folate supplementation and maternal homocysteine levels were monitored across all maternal genotypes at three experimental doses (6.2, 12.4, 24.8 mmole/Kg). Embryonic gene expression changes in the anterior neural tube were also monitored across genotypes of treated and untreated animals using brain specific cDNA microarrays

from the brain molecular anatomy project (BMAP) clone set. Serum folate and homocysteine levels indicated there were both genotype and dose dependant increases in homocysteine and folic acid after supplementation. Gene ontology analyses of expression data indicated an increased expression of specific methyltransferase genes and a downstream methyltransferase cascade. We demonstrate that the selective inhibition of S-adenosyl-L-homocysteine hydrolase (AHCY), which increases SAH levels, or selective inhibition of an identified folate responsive isoprenylcysteine carboxymethyltransferase (ICMT) during murine neural tube closure results in embryos with anterior NTDs. In light of the fact that methyltransferases are downstream acceptors in the active methyl cycle and are inhibited by a low SAM:SAH ratio, we propose that the ability of folic acid to reduce the risk of NTDs is highly dependant on the remethylation cycle and its post-translation methylation of signaling proteins such as Ras.

#### C046

##### IS IT TIME TO RE-EVALUATE METHYL BALANCE IN HUMANS?

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Homocysteine (Hcy) arises from S-adenosylhomocysteine (SAH) which, in turn, is formed when S-adenosylmethionine (SAM) is used as a methyl donor. There are approximately 50 known methyltransferases although bioinformatic analyses of mammalian genomes suggest as many as 300. These methylation reactions constitute a methylation demand which is one of the major determinants of plasma tHcy. Classical work by Mudd suggested that creatine synthesis is the dominant methyltransferase in humans, accounting for about 70% of all SAM utilization. Recently, we have found evidence, in genetically modified mice, that phosphatidylcholine (PC) synthesis by the phosphatidylethanolamine N-methyltransferase (PEMT) is a major determinant of plasma tHcy. PC may be synthesized in liver by two pathways, via PEMT, which uses 3 SAM molecules per PC molecule synthesized, and via the Kennedy pathway, which does not utilize SAM. Pemt knockout mice had plasma tHcy levels that were about 50% lower than controls. On the other hand, liver-specific knockout of CTP: cytidyltransferase- $\alpha$ , a key enzyme of the Kennedy pathway, produced mice with plasma tHcy that were increased by about 30%. We estimate that 30  $\mu$ moles of SAM are required, per day, by a 20 g mouse just for biliary PC production via PEMT. PC synthesis for membrane synthesis and VLDL production will add to this number. We have, therefore, revisited the issue of methyl balance in humans. The spontaneous breakdown of creatine (Cr) and Cr-phosphate to creatinine constitutes a loss of Cr that must be replaced by diet and/or synthesis. Creatinine loss depends on body Cr stores (largely in muscle) and, therefore, varies with age (young>old) and gender (M>F). A 70 kg man, aged 20-39 years, will lose (and must replace) about 1.6 g (16.5 mmol) of creatine per day. We have used the USDA tables of food consumption to calculate dietary Cr intake (in meat and milk) and, employing a bioavailability factor of 90%, have calculated a daily Cr intake, for males of this age group, of 7.9 mmol. Therefore, 8.6 mmol/day must be synthesized. Similar calculations suggest daily rates of Cr synthesis of 5.6 and 3.7 mmol for males in the 40-59 and 60+ age groups, respectively. The corresponding rates for females are about 80% of those found in

males. These rates of Cr synthesis compare with isotopically-measured rates of whole-body transmethylation (TM) of 16-24 mmol/day; these TM rates are relatively unaffected by age and gender. These calculations imply that Cr synthesis is a major, though not dominant, utilizer of SAM-derived methyl groups. Calculated rates of PC synthesis via PEMT, as well as our data with genetically modified mice, suggest that PEMT-flux is at least as great. Since the liver is the principal site of guanidinoacetate methylation to Cr and the exclusive location of PEMT, these considerations imply that the liver is the principal determinant of normal plasma tHcy.

*Funded by CIHR, AHFMR, CDA.*

### C047

#### EFFECT OF HOMOCYSTEINE AND CYSTEINE ON GROWTH AND GENE EXPRESSION OF *SACCHAROMYCES CEREVISIAE*

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**Background.** Intracellular thiol amino acids like cysteine, homocysteine and glutathione play a critical role in the regulation of many important cellular processes like oxidative stress and methylation. Alteration of intracellular thiol concentration has been implicated in many diseased states, for instance, elevated levels of homocysteine is considered to be an independent risk factor for cardiovascular disease and has been associated with Alzheimer's disease.

**Aims.** We wanted to see if yeast could be used as a model system to study the effects of thiols and if so study the change in global gene expression on exogenous addition of thiols.

**Methods.** Varying concentrations of thiols were added to yeast culture and yeast growth was monitored at different time points. Global gene expression was studied using genome wide cDNA microarrays and analysed using commercially available software.

**Results.** We demonstrate that exogenous addition of both cysteine and homocysteine, but not glutathione, inhibits the growth of yeast in a concentration dependent manner. In yeast, unlike in human, cysteine and homocysteine are interconvertible. However, using deletion strains (*str2* delta and *str4* delta) we show that cysteine and homocysteine independently inhibit yeast growth. We also show that exogenous addition of cysteine and homocysteine resulted in upregulation of genes involved in one-carbon metabolism, glycolysis and serine biosynthesis. Interestingly, genes coding for histone proteins that are involved in chromatin assembly were found to be downregulated as were the genes coding for antioxidant enzymes like glutathione peroxidase, catalase and superoxide dismutase. Some of the genes involved in ATP synthesis and transport were also downregulated.

**Conclusions.** Based on the biochemical and gene expression analysis we conclude that exogenous addition of cysteine and homocysteine results in redox stress leading to the alteration of gene expression. Furthermore, cells try to minimize the intracellular concentration of the thiols by upregulating the genes involved in their metabolism.

### C048

#### THE R369C MUTATION IN THE CBS GENE: HIGH POPULATION FREQUENCY AND DECREASED ENZYMIC ACTIVITY

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Recent studies based on mutation screening suggested that homocystinuria due to cystathionine beta-synthase (CBS) deficiency is a more common disorder than originally thought. In Norway the incidence of homocystinuria was estimated to be 1:6.400 (Refsum, *J Pediatr* 2004), which was mostly due to a surprisingly high frequency of c.1105C>T (R369C) allele among newborns. In contrast, the R369C variant was considered a neutral polymorphism by functional study in yeast (Kim, *Hum Mol Genet* 1997), and only three independent alleles carrying this mutation were detected among symptomatic patients worldwide. To further characterize the c.1105C>T (R369C) variant we expressed this mutant in a prokaryotic system to determine its pathogenicity, and we examined its frequency in another European population (i.e. Czechs).

Using PCR-RFLP we observed six c.1105C>T alleles in 600 anonymous newborn samples, which yielded an expected incidence of homozygotes of 1:40.000 (95% CI 1:8.000-1:295.000). These data confirm that frequency of c.1105C>T allele in another European populations is also considerably high, supposedly leading to a high frequency of homozygotes. To test the pathogenicity of the R369C mutant, we employed an *E.coli* expression system followed by activity measurement and native western blotting. Under physiological conditions (expression at 37°C) the mutant enzyme had no detectable activity, and formed aggregates. However, the R369C mutant expressed at 18°C folded into tetramers and exhibited a modest residual activity (~20% of wild type enzyme), which was further activated by S-adenosylmethionine. These data strongly suggests that R369C may be in fact a pathogenic variant with propensity to gain function under folding-permissive conditions.

To conclude, the c.1105C>T (R369C) variant seems to be a pathogenic mutation with high population frequency in Europe. The lack of diagnosed homozygotes, however, leaves the question of clinical consequences of this variant opened.

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## WORKSHOP 7

## RENAL DISEASE - EMERGING ASSOCIATIONS

C049

**HYPERHOMOCYSTEINEMIA INDUCES NITRIC OXIDE PRODUCTION AND PEROXYNITRITE FORMATION IN THE KIDNEY VIA ACTIVATION OF NUCLEAR FACTOR KAPPA-B**

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**Background.** Hyperhomocysteinemia, often found in patients with chronic renal diseases, is emerging as an important risk factor for cardiovascular diseases. Injury of multiple organs including kidney was observed in individuals with hyperhomocysteinemia. The mechanisms by which homocysteine causes kidney injury is still an open question. Activation of nuclear factor kappa B (NF- $\kappa$ B) plays an important role in inflammatory response and can exacerbate organ injury.

**Aims.** The objective of the present study was to investigate the effect of hyperhomocysteinemia on renal NF- $\kappa$ B activation and the consequence of such activation.

**Methods and Results.** Hyperhomocysteinemia was induced in Sprague-Dawley rats after 4 weeks of a high-methionine diet. There was increased phosphorylation of I $\kappa$ B protein leading to NF- $\kappa$ B activation in kidneys of hyperhomocysteinemic rats as determined by Western immunoblotting analysis and EMSA. As a consequence, the expression of inducible nitric oxide synthase (iNOS) mRNA and protein was significantly elevated in these kidneys. Upregulation of iNOS expression resulted in increased nitric oxide (NO) production and peroxynitrite (potent oxygen free radical) formation in kidneys of hyperhomocysteinemic rats. Pretreatment of rats with a NF- $\kappa$ B inhibitor not only abolished NF- $\kappa$ B activation, but also reversed hyperhomocysteinemia-induced iNOS expression in the kidney.

**Conclusions.** Increased iNOS-mediated NO production and peroxynitrite formation via NF- $\kappa$ B activation may represent one of the mechanisms for renal injury during hyperhomocysteinemia.

*This study was supported by Manitoba Health Research Council and Heart and Stroke Foundation.*

C050

**EFFECT OF HOMOCYSTEINE-LOWERING TREATMENT ON PLASMA CONCENTRATIONS OF ASYMMETRIC-DIMETHYLARGININE AND MARKERS OF METHYLATION STATUS IN DIALYSIS PATIENTS**Herrmann W,<sup>1</sup> Obeid R,<sup>1</sup> Schorr H,<sup>1</sup> Knapp J-P,<sup>1</sup> Kuhlmann M<sup>2</sup>*<sup>1</sup>Department of Clinical Chemistry; <sup>2</sup>Department of Nephrology, Saarland University Hospital, Homburg, Germany*

**Background.** Hyperhomocysteinemia, increased plasma concentrations of asymmetric dimethylarginine (ADMA) and disturbed methylation status are common metabolic disturbances in patients with renal failure.

**Aims.** we studied the effect of Hcy-lowering treatment on biomarkers of methylation status and ADMA in dialysis patients.

**Methods.** Patients (n=37) were administered i.v. 5 mg folic

acid plus 0.7 mg B12 and 50 mg B6 thrice a week for 4 weeks. Concentrations of homocysteine (Hcy), S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) and ADMA were measured before and at the end of the trial. Concentrations of Hcy, MMA and cystathionine were followed for 24 weeks after stopping the treatment.

**Results.** Median Hcy was lowered by 50% at the end of the intervention (mean 26.1 vs. 13.2  $\mu$ mol/L) and increased after stopping the treatment to levels comparable to that at start (24.8  $\mu$ mol/L). MMA was lowered by 28% and levels were maintained for 24 weeks after stopping the treatment. Mean concentrations of AdoMet significantly increased at the end of the treatment (125 vs. 136 nmol/L,  $p=0.014$ ). However, this increment was confined to patients whose vitamin B12-status was corrected after the intervention. AdoHcy increased in anemic patients (mean 315 vs. 335 nmol/L;  $p=0.042$ ) and decreased in non-anemic patients (mean 354 vs. 349;  $p=0.068$ ). Improvement in AdoMet/AdoHcy ratio was significant only in non-anemic patients. Concentrations of ADMA were lowered only in patients who had no anemia (mean 1.04 vs. 0.98;  $p=0.023$ ).

**Conclusions.** Short term vitamin treatment may not correct the accompanying metabolic disturbances in uremic patients. The association between Hcy and low AdoMet/AdoHcy ratio is probably not causal. Lowering ADMA may have clinical impact in non-anemic patients. A longer duration of treatment may be considered in future.

C051

**COGNITIVE FUNCTION AND HOMOCYSTEINE IN RENAL TRANSPLANT RECIPIENTS: A FAVORIT ANCILLARY STUDY - DESIGN AND PRELIMINARY REPORT ON CHARACTERISTICS OF THE COHORT**

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**Background.** Renal Transplant Recipients (RTRs) are at high-risk for cerebrovascular disease and stroke, and are likely to be at comparable high risk for subsequent dementia. Hyperhomocysteinemia, which is refractory to folate supplementation, is pervasive in chronic renal insufficiency, even after a successful transplant. However, RTRs are responsive to homocysteine-lowering by high doses of B-vitamins. We are presently conducting a 5 year ancillary study of the cognitive benefits of homocysteine-lowering in an ongoing randomized, controlled, clinical trial on the effect of a high-dose combination of folic acid, vitamin B12 and vitamin B6 on arteriosclerotic cardiovascular disease outcomes in chronic, stable RTRs, the Folic Acid for Vascular Outcome Reduction in Transplantation (FAVORIT).

**Aims.** To prospectively determine the efficacy of homocysteine-lowering treatment for preventing cognitive decline and to characterize cognitive function in relation to homocysteine and other risk factors for vascular disease in RTRs who represent a high-risk, non-demented population.

**Methods.** The study is designed to measure blood biochemical and cognitive outcomes using a face to face battery of neuropsychological tests and a telephone interview for cognitive status at baseline and after a 3-4 follow up in 1000 participants in the FAVORIT trial aged >49 years.

**Results.** Enrollment in the ancillary study has begun with randomization for the parent clinical expected to be completed in 2006. Preliminary analysis of cognitive scores from the first 79 subjects shows above average intelligence, normal memory function and impaired executive function,

## Homocysteine Metabolism

attention and mental processing speed as well a high prevalence of depressive symptoms.

**Conclusions.** Limited preliminary results indicate that RTRs have some degree of cognitive impairment. Given that renal insufficiency represents accelerated degenerative changes in vascular and possibly other systems, it is hoped that the outcome of this study will be of importance for understanding the potential of homocysteine lowering treatment for cognitive protection in the general population.

### C052

#### EFFECT OF FOLIC ACID ON METHIONINE AND HOMOCYSTEINE METABOLISM IN END-STAGE RENAL DISEASE

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The pathogenesis of hyperhomocysteinemia in end-stage renal disease (ESRD) is unclear. Folic acid lowers, but does not normalize, the plasma homocysteine level in patients with ESRD, but its effect on whole body metabolism of homocysteine is unknown. In this study, we aimed to elucidate the apparent folate resistance in ESRD. We studied the effect of 3 weeks of oral treatment with 5 mg folic acid per day on homocysteine metabolism in six chronic hemodialysis patients and six healthy controls. Primed, continuous infusions with [2H3-methyl-1-13C]methionine were used to determine flux rates of methionine transmethylation, homocysteine remethylation, and homocysteine transsulfuration. Metabolic homocysteine clearance was defined as the ratio of transsulfuration and plasma homocysteine level. Results are presented in the table. Folic acid treatment lowered plasma homocysteine significantly by 39% (95% confidence interval (CI): 5 to 73) in the ESRD group, but plasma homocysteine remained higher than baseline values in the control group. In ESRD patients, homocysteine remethylation and methionine transmethylation rate increased by 34% (95% CI: 5 to 62) and 22% (95% CI: 5 to 39), respectively, i.e. levels that were similar to the baseline values of the control group. Transsulfuration rate and metabolic homocysteine clearance were not significantly altered by folic acid treatment in both the ESRD and the control group. In conclusion, in ESRD patients, folic acid treatment lowers, but does not normalize plasma homocysteine, whereas homocysteine remethylation and methionine transmethylation increase to levels found in untreated healthy controls. These findings indicate a persistent, folate-independent, defect in metabolic homocysteine clearance in ESRD.

**Table 1. Plasma homocysteine concentrations, one-carbon flux rates, and metabolic homocysteine clearance before and after folic acid treatment in six patients with end-stage renal disease and six healthy controls.**

Characteristic	ESRD		Control	
	Baseline	After folic acid	Baseline	After folic acid
Plasma homocysteine (μmol/L)	38.7±14.8	20.2± 4.1*	8.1±1.4	6.5±1.0*
Remethylation (μmol/kg FFM/hour)	3.9±1.4	5.1±2.1*	5.0±1.7	6.7±1.1
Transmethylation (μmol/kg FFM/hour)	6.7±1.7	8.0±1.9*	8.1±1.7	9.0±1.8
Transsulfuration (μmol/kg FFM/hour)	2.8±0.7	2.9±1.0	3.2±0.9	2.7±1.1
Metabolic homocysteine clearance (L/kg FFM/hour)	0.09±0.05	0.15±0.05	0.41±0.14	0.43±0.18

Values are mean ± SD. \*P < 0.05, \*P < 0.01 and \*P = 0.055 compared to baseline. Abbreviations: ESRD, end-stage renal disease; FFM, fat-free mass.

### C053

#### HOMOCYSTEINE IS DIRECTLY ASSOCIATED WITH SOLUBLE VASCULAR CELL ADHESION MOLECULE 1 (VCAM-1) IN TYPE 1 AND TYPE 2 DIABETES

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**Background.** Elevated levels of VCAM-1 in plasma are indicative of endothelial dysfunction and are associated with microangiopathy, macroangiopathy, and cardiovascular mortality in diabetes patients. Homocysteine, a risk factor for vascular disease, is often elevated in diabetic patients, usually as a result of renal insufficiency. Homocysteine induces VCAM-1 expression and increases monocyte adhesion in human aortic endothelial cells. Similar observations have been reported in rats and mice fed hyperhomocysteinemic diets. The cross-sectional association between homocysteine and VCAM-1 in diabetic patients, however, has not been studied.

**Aims.** To assess the association between homocysteine and VCAM-1 in patients with Type 1 and Type 2 diabetes.

**Methods.** In a study of cross-sectional design, Type 1 (n=37) and Type 2 (n=73) diabetic patients were recruited, along with healthy controls of similar age and sex (n=40). Total plasma homocysteine was determined by HPLC; folate and vitamin B12 by radioassay; pyridoxal-5'-phosphate (PLP) by radioenzymatic assay; creatinine by standard spectrophotometric assay; and VCAM-1 by ELISA.

**Results.** Homocysteine was significantly higher in both the Type 1 and Type 2 patients compared with the controls (11.5±7.3 and 11.1±5.5 vs 8.9±3.0 μmol/L, respectively; p<0.05), as was VCAM-1 (587±249 and 562±294 vs 359±158 ng/mL, respectively; p<0.001). Within the diabetes groups and the control group, homocysteine was directly associated with VCAM-1 (p<↑0.006). The associations remained significant in both diabetes groups (p<↑0.04), but not the control group, after controlling for confounding by age, sex, folate, B12, PLP, and creatinine.

**Conclusions.** Homocysteine is directly associated with VCAM-1 in diabetic patients. Whether reducing homocysteine in these patients with B vitamin supplements will reduce VCAM-1 levels and the risk of vascular disease and related morbidity and mortality remains to be determined.

**Funding.** Diabetes Action Research and Education Foundation.

### C054

#### INCREASED BONE RESORPTION IN THE PRESENCE OF ELEVATED HOMOCYSTEINE LEVELS

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Prevention of osteoporosis is a major public health issue. Recently, increased plasma homocysteine (HCY) has been suggested as a risk factor for osteoporotic fractures. It can be speculated that HCY adversely affects bone metabolism by stimulating bone resorption. This study aimed to analyze the effect of HCY on bone resorption. First, we investigated the relation between HCY and bone metabolism in 143 peri-

postmenopausal women (66±12 years) measuring circulating HCY, osteocalcin (OC), crosslinks (CTX), osteopontin (OPG) and soluble receptor activator of NF- $\kappa$ B ligand (sRANKL) levels. Additionally, we analyzed urinary desoxypyridinoline crosslinks (DPD) and bone mineral density (BMD) at lumbar spine (BMD-LS) and total hip (BMD-HIP). Second, we cultured PBMCs from 19 healthy subjects (mean age: 30±5 years, mean HCY: 9.5±1.9  $\mu$ mol/L) to obtain fully differentiated osteoclasts (OC). Culture medium was spiked with 0, 10, 50, 100  $\mu$ mol/L HCY. OC activity was quantified by TRAP and cathepsin K (CPK) activity measurement. According to BMD subjects of the clinical trial were classified as normal (n=24), osteopenic (n=51) and osteoporotic (n=68). Median HCY did not change with increasing BMD. Contrary, serum levels of the bone resorption marker DPD increased with increasing quartiles of HCY ( $p=0.043$ ), while levels of OC, OPG and sRANKL did not. Partial correlation analysis after correction for creatinine and age confirmed an association between HCY and DPD ( $r=0.175$ ,  $p=0.038$ ). No relations were seen between serum levels of HCY and OC, OPG, sRANKL, BMD-LS and BMD-HIP. The in-vitro experiments showed that TRAP activity increased dose dependently with increasing HCY concentrations ( $p<0.001$ ). HCY levels of 10, 50 and 100  $\mu$ mol/L stimulated TRAP activity between 15% and 42%. Additionally, HCY stimulated CPK activity ( $p=0.005$ ). In the presence of 100  $\mu$ mol/L HCY CPK activity was about 38% higher than in controls ( $p=0.002$ ). Our data suggest increased bone resorption in the presence of elevated HCY, which is possibly mediated by osteoclast activation. The relation between HCY and bone resorption was independent from OPG and sRANKL.

#### C055

##### METHOTREXATE INCREASES SKELETAL MUSCLE GLUT4 EXPRESSION AND IMPROVES METABOLIC CONTROL IN EXPERIMENTAL DIABETES

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**Background.** Long-term administration of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) mimics the effects of endurance exercise, activating AMP-kinase and increasing skeletal muscle expression of GLUT4 glucose transporter, a key actor in glucose utilization and homeostasis. *In vivo*, tissue concentrations of AICAR, a naturally occurring intermediate in the purine *de novo* synthesis, can be increased by low doses of the antifolate drug methotrexate (MTX), through the inhibition of the enzyme AICAR transformylase.

**Aims.** In this study, we verified whether chronic treatment with low doses of MTX would increase skeletal muscle GLUT4 expression and improve metabolic control in an experimental model of type 2 diabetes.

**Methods.** MTX (0.5 mg/kg body weight) or vehicle was administered intraperitoneally once a week for 4 weeks to twelve genetically diabetic female C57BL/KsJ-m+/+Leptdb mice (db+/db+) and twelve normoglycemic littermates (db+/+m). At the end of each treatment period, animals were sacrificed and skeletal muscle tissue was removed,

snap-frozen and stored at -80°C for total GLUT4 mRNA and protein content measurements.

**Results.** In the db+/db+ mice, MTX treatment was associated with a 2 fold increase in skeletal muscle GLUT-4 protein concentration ( $p<0.01$ ) and a >4 fold increase in GLUT4 mRNA expression ( $p<0.01$ ), as compared to vehicle-treated mice; no significant differences were noted in controls. MTX treatment was associated with a significant reduction of both glucose and insulin serum concentrations in diabetic mice ( $p<0.001$ ), and a decrease of glucose levels only ( $p<0.05$ ) in controls.

**Conclusions.** Our data show that chronic treatment with low doses of MTX is able to increase skeletal muscle GLUT4 expression and to improve metabolic control in experimental type 2 diabetes, suggesting an alternative route to activate AMPK-downstream cascade, through the inhibition of the enzyme AICAR transformylase.

#### C056

##### POLYMORPHISMS IN FOLATE-RELATED GENES AND RISK OF PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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**Background.** The folate status may modulate the risk of childhood ALL.

**Aims.** we investigated the presence of common polymorphisms in genes involved in folate metabolism, which may influence the susceptibility to ALL.

**Methods.** DNA was isolated from 245 pediatric ALL patients (cases) at the time of diagnosis and from 184 pediatric patients treated for non-hematological diseases (controls). Polymorphisms in methylenetetrahydrofolate reductase (MTHFR 677C>T, MTHFR 1298A>C), methionine synthase (MTR 2756A>G), methionine synthase reductase (MTRR 66A>G), methylenetetrahydrofolate dehydrogenase (MTHFD1 1958G>A), serine hydroxymethyl transferase (SHMT1 1420C>T), thymidylate synthase (TS 2R3R), and the reduced folate carrier (RFC 80G>A) were detected by PCR-RFLP or real-time PCR.

**Results.** In ALL patients, an increased occurrence was observed of the RFC 80GA variant (odds ratio [OR]=1.55; 95% confidence interval [CI], 1.01-2.39;  $p=0.05$ ) and the RFC 80AA variant (OR=1.78; 95% CI, 1.02-3.09;  $p=0.04$ ). Likewise, the SHMT1 TT genotype showed an OR of 1.96 (95% CI, 0.93-4.12;  $p=0.08$ ).

**Conclusions.** The RFC 80A allele or the SHMT1 TT genotype are associated with increased risk of ALL.

**WORKSHOP 8**  
**MECHANISMS of HOMOCYSTEINE**  
**TOXICITY - ANIMAL MODELS -**  
**THE HOMOCYSTINURIAS**

**C057****MOLECULAR TARGETING OF METALLOTHIONEIN BY L-HOMOCYSTEINE AND IMPAIRMENT OF ZINC-BINDING CAPACITY**

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**Background.** Elevated plasma total homocysteine (tHcy) is an independent risk factor for occlusive vascular disease. Due to the highly reactive nature of homocysteine's thiol and the limited capacity of the vasculature to metabolize it, homocysteine is likely to target cysteine-rich molecules to form mixed disulfide conjugates, leading to altered function of the targeted molecule.

**Aims.** We hypothesized that metallothionein (MT), an essential zinc-chaperone for multiple biochemical processes, is an intracellular target for homocysteinylation because of its extremely rich cysteine content used in the coordination of zinc.

**Methods.** In this study, the extent of MT homocysteinylation was determined to see whether this mixed disulfide conjugate alters zinc binding in human aortic endothelial cells (HAEC). Cultured HAEC were incubated with 35S-L-homocysteine. Autoradiography and Western blotting were used to demonstrate homocysteinylation of MT and confirmed by MT antibody-binding experiments. The loss of zinc-binding capacity was determined using zinc-chelating Sepharose.

**Results.** These studies provide the first evidence that homocysteine targets MT. Moreover, the targeting of MT by homocysteine impairs the zinc-binding function of MT. Because MT is essential for delivery of zinc to superoxide dismutase, interference with the ability of MT to bind zinc by homocysteine will lead to increased levels of reactive oxygen species and oxidative stress in HAEC. This was confirmed by treating cells with 5-(and-6)-chloromethyl-2', 7'-dichlorodihydro-fluorescein diacetate, acetyl ester and fluorescence microscopy.

**Conclusions.** the pathological consequences of molecular targeting of MT by homocysteine represent a novel mechanism for homocysteine-mediated vascular damage. In addition, due to the importance of zinc in signaling and enzymatic function, the interference of the zinc-binding function in homocysteinylation of MT could have deleterious consequences across multiple biochemical processes.

**C058****EFFECT OF FOLIC ACID ON ADMA, NO AND INDICES OF VESSEL FUNCTION IN HYPERHOMOCYSTEINEMIC SUBJECTS**Stanger O,<sup>1</sup> Paulweber B,<sup>2</sup> Matteucci-Grothe R,<sup>3</sup> Fowler B,<sup>4</sup> Pachinger O,<sup>5</sup> Hufnagl C,<sup>1</sup> Stühlinger M<sup>5</sup>

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**Background.** Endothelial function is impaired in hyperho-

mocysteinemic subjects. Homocysteine inhibits NO production, in part, by accumulating asymmetric dimethylarginine (ADMA) in endothelial cells.

**Aims.** We investigated the effect of oral folic acid treatment on ADMA and NO in a cohort of healthy adults with hyperhomocysteinemia. Changes in vascular function were monitored. Methods 64 subjects (57±4 years, Hcy>12 μmol/L) free of vascular disease were randomized in a double-blinded fashion to receive either placebo or 0.4mg, 1mg and 5mg folic acid for eight weeks. Fasting blood samples were taken every two weeks. Healthy control subjects with normal homocysteine concentrations were included to compare baseline ADMA and homocysteine levels.

**Results.** Characteristics of randomized study populations were not different. There were significant correlations at baseline between homocysteine and ADMA ( $p=0.003$ ), folic acid, arginine ( $p=0.05$ ), arginine/ADMA ratio ( $p=0.002$ ) and also with diastolic ( $p=0.04$ ) and mean arterial pressure ( $p=0.02$ ). All doses of oral folic acid significantly reduced plasma homocysteine concentrations after two weeks, whereas placebo did not affect homocysteine. ADMA plasma concentrations were not affected by placebo or folate at any dose or time. NO bioavailability was significantly improved in the treatment group with 5mg of folic acid (19.16 s. 24.80 vs. 29.31). No significant changes were seen for systemic and total vessel resistance and for the elasticity index of small and greater vessels.

**Conclusions.** In this randomized, placebo-controlled trial oral low-dose and high-dose folic acid caused significant reductions of plasma homocysteine and improved NO bioavailability in the 5 mg treatment group only. The decline of homocysteine and the changes in NO were not accompanied with decrements of ADMA. The baseline correlation between homocysteine and ADMA suggest a more complex association that is unaffected by short-term vitamin supplementation.

**C059****FUNCTIONAL CHARACTERIZATION OF 14 CBS MUTATIONS FOUND IN HOMOCYSTINURIC PATIENTS**Urreiziti R,<sup>1</sup> Asteggiano C,<sup>1</sup> Cozar M,<sup>1</sup> Frank N,<sup>2</sup> Vilaseca MA,<sup>3</sup> Grinberg D,<sup>1</sup> Balcells S<sup>1</sup>

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Classical homocystinuria (OMIM \*236200) is an autosomal recessive disease due to cystathionine beta-synthase (CBS; EC4.2.1.22) deficiency. It is characterized by high levels of homocysteine and methionine in plasma and urine. The worldwide incidence for this disease is estimated at 1/335.000 live births, although there is a wide range of variation depending on the population. The CBS gene was mapped to 21q22.3. More than 130 different mutations have been described until now and the activities of 33 of these were analyzed in an E.coli expression system (CBS Mutation Database: <http://www.uchsc>). The aim of this study was to analyze the enzymatic activity of 14 mutations found in our patients' cohort. The cDNA of the CBS gene, cloned in the pkk3.88 plasmid was directly mutated generating these 14 alleles. The mutations were expressed in the E.coli strain XL-Blue and their enzymatic activity was measured in an in-vitro reaction. Besides, these expressions were tested in SDS-PAGE and in non-denaturing PAGE followed by Western blot. The 14 mutations characterized were: R125W (c.373C>T), G148R (c.442G>A), M173V (c.517A>G),

T191M (c. 572C>T), A226T (c. 676G>A), C275Y (c.824G>A), R336C (c. 1006C>T), R336H (c. 1007G>A), L338P (c.1013T>C), S349N (c.1046G>A), R379Q (c. 1136G>A), L456P (c.1367T>C), G522fsX540 (c.1566delG) and R548Q (c.1643G>A). Eleven of them showed an activity lower than 5% of the wt protein. In contrast, mutations A226T and M173V presented near 20% of the wt activity, whereas the activity of R548Q was higher than 68% of wt. This activity, together with the fact that this change was found in a control chromosome, suggest that it is a new polymorphism rather than a pathogenic mutation. In the majority of cases, the mutated protein showed a decreased signal in Western blot analysis compared with the wt. The non-denaturing PAGE showed that the wt protein kept the capacity to form a multimeric quaternary structure, whereas in two mutations this structure grade was dramatically reduced and it was completely absent in the rest of them.

## C060

### MOUSE MODELS OF CBS DEFICIENT HOMOCYSTINURIA: FROM MICROARRAYS TO A NOVEL IMPROVED TREATMENT

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Cystathionine beta-synthase (CBS) deficient homocystinuria (CBSDH) exerts pleiotropic effects upon a wide range of tissues. The pathological mechanisms that underlie CBSDH are poorly understood. Using a novel homocystinuric mouse model and normal control mice, we examined changes in the hepatic expression profiles of greater than 39,000 mouse genes using the Murine Genome mouse expression chip MOE 430A from Affymetrix. After a process of rigorous data management and target validation, we found a total of 235 genes were significantly down-regulated compared to controls while 290 genes were significantly up-regulated.

The data presents a coordinated and interrelated picture of oxidative stress (25 genes) acting to induce genes involved in ER stress/growth arrest (18 genes), lipid biosynthesis (25 genes), and NF-kappaB/TNF-alpha mediated inflammation and acute phase response proteins (25 genes). This analysis prompted the hypothesis that the some of the clinical sequelae of CBSDH are caused by oxidative stress acting both directly and indirectly through TNF-alpha/NF-kappaB mediated inflammation and ER stress.

Good evidence exists showing that taurine can suppress both TNF-alpha production and NF-kappaB activation, decrease inflammation, is tissue-protective in oxidant-induced injury and can also act as a chemical chaperone acting to ameliorate ER stress. We treated a group of hypercoagulative homocystinuric mice (n=6) with either taurine or betaine in drinking water. After treatment, all of these mice and an age-matched group of untreated homocystinuric mice were tested for tail bleeding times. In contrast to betaine, which at best, only partially ameliorated hypercoagulation, taurine treatment completely abolished the hypercoagulative phenotype of the homocystinuric mice. The therapeutic effects of taurine showed superior persistence compared to betaine without significantly changing either Hcy or cysteine levels. Taurine treatment had no effect upon coagulation times in normal mice. Taken together, these results indicate that taurine therapy has excellent potential to improve existing treatments of CBSDH in humans.

## C061

### OXIDATIVE STRESS CAUSES ALDEHYDE ADDUCTION OF PROTEINS IN CYSTATHIONINE BETA-SYNTASE DEFICIENT HOMOCYSTINURIC MICE

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The pathogenic mechanisms that underlie the clinical sequelae of Cystathionine beta-synthase (CBS) deficient homocystinuria (CBSDH) are poorly understood. New mouse models of CBSDH indicate that many aspects of the pathology of this disease are due to oxidative stress caused by elevation of the prooxidant Hcy and a concomitant decrease in antioxidant capacity. Oxidant stress can cause the autocatalytic process of lipid peroxidation, the principal alpha, beta-unsaturated aldehydic products of which, 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) can be cytotoxic. We report elevated levels of MDA in plasma samples from CBSDH mice. MDA and 4-HNE can impair protein function by forming covalent adducts with solvent accessible cysteine, histidine and lysine residues. We performed Immuno detection of MDA and 4-HNE adducted proteins using immunoglobulin G-purified polyclonal rabbit antibodies specific for either 4-HNE or MDA-adducted protein epitopes. Our analysis found striking evidence of both 4-HNE and MDA adducted proteins in the tissues of CBS deficient mice. The MDA and 4-HNE adducted proteins show a different intracellular distribution from each other indicating that each aldehyde is adducting different populations of proteins in these tissues. No detectable aldehyde adduction was found in wild type mice controls. The degree of adduction found in the livers of moribund CBSDH mice has never been reported previously and is so severe that it is virtually certain to be contributing to the profound hepatopathy incurred by these mice. The susceptibility of cysteine, lysine and histidine residues to aldehyde adduction indicates that proteins involved with cross-linking and/or complex folding pathways such as Fibrillin-1 and collagen could be particularly vulnerable to impairment by chronic exposure to MDA or 4-HNE adduction. Our findings represent definitive proof of oxidative stress in CBSDH and suggest a possible novel mechanism for the connective tissue disturbances typically associated with this disorder.

## C062

### CONTRASTING ENZYMIC BEHAVIOR OF TWO MUTANT CBS PROTEINS ASSOCIATED WITH PYRIDOXINE RESPONSIVE HOMOCYSTINURIA

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CBS deficiency is a recessive genetic disorder characterized by extremely elevated levels in plasma homocysteine. About half of all CBS deficient patients respond clinically to pharmacologic doses of pyridoxine, the precursor of 5'-pyridoxal phosphate (PLP) a co-factor for the enzyme. Several patients homozygous for either I278T or R266K have been shown to be pyridoxine responsive. Here, we have performed a biochemical and enzymatic characterization of these mutant enzymes. Using serine and homocysteine as substrates, we found that recombinant R266K has about 30% the specific activity of the wild type enzyme, while I278T only has about 1% activity. Kinetic studies show that the decreased activity in both enzymes is due to decreased turnover rate and not substrate binding. We have also examined affinity of PLP to these enzymes. R266K, but not I278T, has about a three-fold

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reduced affinity toward PLP compared to the wild-type version of the enzyme. This is consistent with complementation studies in *S. cerevisiae*, that demonstrate that yeast expressing R266K need increased pyridoxine in the media in order to grow. The low level of enzyme activity and the lack of response to PLP for I278T *in vitro* suggested that the enzyme might be functioning differently *in vivo*. Therefore, we have made a mouse that is deleted endogenous mouse Cbs and expresses the human I278T CBS gene under control of the human metallothionein promoter. Western analysis shows that the I278T enzyme is expressed in the liver at levels comparable to the wild-type human CBS protein. However, plasma homocysteine levels in these animals are extremely elevated and do not respond to large doses of pyridoxine. Thus the mouse data is entirely consistent with the *in vitro* behavior of the enzyme. These studies indicate that the pyridoxine response associated with the I278T mutation in humans may have a more complicated explanation.

### C063

#### HYPERHOMOCYSTEINEMIA CAUSES ATHEROGENIC HDL METABOLISM IN CBS/APOE MICE

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Hyperhomocysteinemia (HHcy) is an important non-lipid risk factor for cardiovascular disease and is associated with increased aortic lesions and decreased plasma HDL-cholesterol (HDL-C) in mice (1). The mechanism for this association is unknown. In this study, we found that HHcy is significantly and negatively correlated with plasma HDL-C and apoA1 protein levels in patients with coronary heart disease (CHD). To study the underlying mechanisms, we employed mice with targeted deletions of the genes for apolipoprotein E (apoE) and cystathionine- $\beta$ -synthase (CBS). HDL-C decrease in severe HHcy was caused by the loss of large HDL particles. HDL composition analysis revealed an increase in the free cholesterol ratio and a decrease in the total protein ratio. Mouse plasma from CBS/apoE deficient mice attenuated cholesterol efflux from cholesterol  $\beta$ -loaded macrophages. ApoA1 protein levels were decreased in the liver and plasma of HHcy

mice, and this is correlated with reduced plasma lecithin:cholesterol acyltransferase fractional activity against endogenous substrates. In addition, severe HHcy resulted in faster clearance of HDL-cholesterol ester that correlated with elevated hepatic SR-B1 protein levels. These results suggest that decreased apoA1 synthesis and increased HDL-cholesterol clearance contribute to HDL reduction in HHcy.

### C064

#### HYPERHOMOCYSTEINEMIA IMPAIRS ENDOTHELIAL FUNCTION BY NITRIC OXIDE INHIBITION AND PKC ACTIVATION IN CBS MICE AND AORTIC ENDOTHELIAL CELLS

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Hyperhomocysteinemia (HHcy) is an independent risk factor for cardiovascular disease (CVD), and is associated with endothelial dysfunction. However, the mechanism for homocysteine (Hcy)-induced endothelial dysfunction is largely unknown. In this study, we examined the role of HHcy in endothelial dysfunction using two functional models, aortic rings and intravital videomicroscopy of the cremaster, in cystathionine- $\beta$ -synthase (CBS) null mice. We found that arterial relaxation in response to acetylcholine, an endothelium-dependent vessel relaxant, was significantly impaired in severe HHcy. Impaired endothelium-dependent relaxation was restored by a nitric oxide donor, but not by superoxide dismutase and catalase. Plasma levels of asymmetric dimethylarginine were not increased in CBS<sup>-/-</sup> mice. Endothelial nitric oxide synthase (eNOS) activity was significantly reduced in aortic endothelial cells from CBS<sup>-/-</sup> mice, as well as in Hcy-treated mouse and human aortic endothelial cells. This was correlated with decreased protein expression and increased serine 495 phosphorylation of eNOS. Hcy-mediated eNOS inhibition was not rescued by adenoviral-transduction of superoxide dismutase and glutathione peroxidase, or by tetrahydrobiopterin and arginine supplementations. The protein kinase C (PKC) inhibitor GF109203X reversed Hcy-mediated eNOS inactivation and serine 495 phosphorylation. These data indicate that HHcy impairs endothelial function and eNOS activity through PKC activation.

## POSTERS

## P001

**MOUSE MODELS OF CBS DEFICIENT HOMOCYSTINURIA ARE IN A HYPERCOAGULATIVE STATE: TREATMENT EFFICACY OF BETAINES VARIES SIGNIFICANTLY AS A FUNCTION OF TIME**

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Cystathionine beta-synthase (CBS) deficient homocystinuria (CBSDH) is accompanied by mental retardation, thromboembolic complications and a range of characteristic connective tissue disorders. Currently, the only effective treatment for pyridoxine non-responsive individuals with CBSDH is methionine restriction and betaine therapy. Ethical considerations and the absence of a suitable animal model have precluded more detailed studies of the long-term metabolic adaptive effects of this therapy. If the therapeutic effects of betaine can be maximized sufficiently, it is conceivable that either the cost of the treatment could be significantly reduced or the requirement for methionine restriction could be relaxed leading to a significant increase in quality of life for individuals with this disease. We present a new mouse model of CBSDH that is in a hypercoagulative state as determined by tail bleeding times and determination of partial thrombin time. Treatment of these mice for one week with betaine resulted in effective lowering of plasma homocysteine (Hcy) and S-adenosylhomocysteine (SAH) levels. These changes were accompanied by a significant lengthening of tail bleed times indicating that this treatment ameliorates the hypercoagulative phenotype. After one month of treatment, the same mice showed evidence of a metabolic adaptation resulting in a rise in plasma Hcy and SAH levels. Although plasma levels of these compounds was still significantly lower than that observed in untreated CBSDH mice, tail bleeding times returned to a level comparable with untreated controls. These findings constitute the first report of a hypercoagulative phenotype in a mouse model of CBSDH and indicate that there is a relatively sharp transition in terms of plasma Hcy levels and the severe increases in thrombotic risk associated with this disease.

## P002

**COMPENSATORY MECHANISMS TO OXIDATIVE STRESS IN YOUNG ADULT RATS FOLLOWING FOLATE DEPLETION**

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**Background.** Folate plays a fundamental role as methyl donor in amino acid and nucleic acid metabolisms. Folate deficiency is associated with increased risk of degenerative diseases such as occlusive vascular diseases, cognitive and neurological dysfunction and cancers. To date, no causal relationship between insufficient folate status and the aeti-

ology of those diseases has been demonstrated. Particularly, more data are needed to explain the cellular and molecular mechanisms that underlie the metabolic changes induced by folate-deficiency.

**Aims.** To determine the effects of folate deficiency on the proteome of liver, the main tissue of folate storage and metabolism.

**Methods.** Four month-old rats were fed for 4 weeks an amino acid-defined diet containing 1.5 (control) or 0 mg (folate-depleted) folic acid/kg. Plasma and liver folate concentrations were assessed by microbiological assay and homocysteinemia by HPLC. Different markers of oxidative stress and indicators of the antioxidant response were also measured. A proteomic approach (2D electrophoresis and MALDI-TOF mass spectrometry) was used to investigate the changes in abundance of hepatic proteins after folate deficiency.

**Results.** Folate deprivation decreased plasma and hepatic folate concentrations and increased homocysteinemia. It also led to diminished plasma levels of vitamin E and Ferric Reducing Ability of Plasma (FRAP), while Thiobarbituric Reactive Substances (TBARS), an index of lipid peroxidation, were augmented in both liver and heart. Proteomics allowed the identification of 9 differentially expressed hepatic proteins when comparing folate-deficient to control rats. Up-regulated proteins included glutathione peroxidase 1, one of the enzymes involved in the response to oxidative stress. Decreased abundance was observed for caspase 1 and pre-proalbumin which play a role in the inflammatory response, and for cofilin 1 which has been associated with tumorigenesis.

**Conclusions.** Our results highlight several hepatic proteins that respond to folate deficiency and some may be part of compensatory mechanisms to the concurrent oxidative stress.

**Table 1. Folate status, homocysteinemia, and biochemical markers of oxidative stress of rats fed a folate-depleted diet or a control diet.**

	Control	Folate-depleted
<b>Plasma</b>		
Folate, nmol/L	159.6±12.7	14.1±1.1*
Homocysteine, $\mu$ mol/L	6.4±1.8	22.9±8.6*
Vitamin E, $\mu$ mol/L	23.3±5.6	16.9±3.8*
FRAP, $\mu$ mol/ml	218.6±21.0	185.7±23.3*
<b>Heart</b>		
TBARS, nmol/g wet tissue	118.5±12.52	180.59±35.35*
<b>Liver</b>		
Folate, nmol/g wet tissue	20.5±2.2	4.0±1.0*
TBARS, nmol/g wet tissue	166.9±17.5	201.9±15.9*

Values are means  $\pm$  SD. n = 8 (FRAP and Vitamin E), n = 7 (Folate and homocysteine), n=6 (TBARS). \*Significantly different from control according to Mann-Whitney or Student t test (P<0.05). FRAP: Ferric Reducing Ability of Plasma. TBARS: Thiobarbituric Reactive Substances.

P003

**CYTOPLASMIC SERINE HYDROXYMETHYLTRANSFERASE REGULATES THE HOMOCYSTEINE REMETHYLATION PATHWAY**

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Serine hydroxymethyltransferase catalyzes the reversible interconversion of serine and tetrahydrofolate (THF) to glycine and methyleneTHF. Mammals express both cytoplasmic and mitochondrial isozymes that are encoded on separate genes. The mitochondrial isozyme (mSHMT) is expressed ubiquitously and functions to generate one-carbons from serine for cytoplasmic folate-mediated one-carbon metabolism. The cytoplasmic isozyme (cSHMT) displays tissue-specific expression and is believed to regulate cytoplasmic one-carbon metabolism. Tissues expressing cSHMT include liver, kidney and the crypts of the ileum and colon. We have generated mice that lack expression of cSHMT in the germ line, and mice that lack cSHMT expression specifically in the liver. Mice lacking cSHMT in the germ line are fertile and viable but display altered homocysteine metabolism and 3 to 5-fold elevations in the hepatic SAM/SAH ratio. Loss of cSHMT expression exclusively in the liver does not affect the hepatic SAM/SAH ratio, indicating the inhibitory effect of cSHMT on the homocysteine remethylation cycle may not occur in this tissue. The data support previous findings in cell culture models that cSHMT represses homocysteine remethylation (Herbig *et al.*, 2002, *J Biol Chem* 277:38381-90), and that alterations in cSHMT expression could impact cellular methylation reactions.

P004

**BEHAVIORAL PHENOTYPE OF ADULT-ONSET HOMOCYSTEINEMIA IN AGED MICE WITH CONDITIONAL CBS DEFICIENCY**

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*Background.* Homocysteinemia is associated with age-related cognitive decline and dementia in humans but the mechanisms underlying this association are uncertain. Targeted deletion of the Cystathionine Beta Synthase gene produces severe homocysteinemia in mice together with developmental abnormalities that render them unsuitable for behavioral research. A new mouse model (tg hCBS) in which the murine CBS gene is replaced by an inducible human CBS gene develops normally but expresses severe inducible homocysteinemia.

*Aims.* To characterize the behavioral phenotype of tg hCBS mice with chronic induced adult-onset homocysteinemia.

*Methods.* Male and female 20-22 month old tg hCBS mice were used that had been back-crossed for 2-3 generations and that had been exposed to at least 16 months of severe homocysteinemia. Mice underwent behavioral testing including psychomotor tests, the elevated plus maze and light-dark shuttle box tests of anxiety, the novel-object recognition memory test and novel environment exploration and home cage activity measurements. Age-matched male C57Bl/6 wild type mice were used as a behavioral reference group. Mice were euthanized and brains removed for histopathology.

*Results.* Subtle behavioral differences were observed

between the two groups on the light-dark test, novel object recognition, and novel environment exploration and home cage activity. Brain histopathology did not reveal gross anatomical lesions.

*Conclusions.* Aged tg hCBS mice appear to express subtle differences in behavior compared with a standard reference model of mouse aging. They do not display gross neurodegeneration. The tg hCBS mouse is a promising model for elucidating the relationship between brain, behavior and homocysteine metabolism.

P005

**SUSTAINED REDUCTION OF SERUM HOMOCYSTEINE BY NAKED PLASMID DNA GENE DELIVERY OF CYSTATHIONINE BETA-SYNTASE**

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*Background.* Elevated circulating homocysteine is an independent risk factor for the development of cardiovascular and other diseases. Cystathionine beta-synthase (CBS) is a key enzyme responsible for the metabolism of homocysteine. Genetic variation results in hyperhomocysteinemia in 10% of humans. Naked DNA delivery consists of injecting plasmid DNA in a saline solution into muscle or dermal tissue. Cells at the site of injection are transfected and produce the encoded protein. Unlike viral vectors, DNA injection does not induce immune responses against the vector and does not integrate into the genome.

*Aims.* To demonstrate that CBS gene delivery can lower homocysteine concentrations in normal and knockout mice and thus ameliorate pathology in homozygous CBS knockout animals.

*Methods.* Plasmid DNA encoding mouse CBS was injected once into normal or transgenic CBS knockout mice. Control animals were injected with a beta-galactosidase plasmid. Homocysteine levels were measured in serum samples. Animals were weighed every 3 days after treatment.

*Results.* In our initial experiments, serum homocysteine decreased by 10% one week after injection of CBS and 25% by 20 weeks in wild type animals. This lower level was maintained for the durations of the experiment (36 weeks). Treated homozygous knockout animals showed a 40% higher relative weight gain than untreated animals between 3 and 7 weeks of age.

*Conclusions.* We have achieved sustained reductions of serum homocysteine with a single treatment of plasmid DNA. Treatment appears to enhance the general health of CBS knockout animals. Reductions of homocysteine levels by 30% could have significant health benefits in humans. This approach may also prove useful for studies of homocysteine metabolism under conditions in which metabolic flux through this pathway is increased by methionine loading.

## P006

**REFERENCE INTERVALS OF VITAMIN B6 IN A GROUP OF VOLUNTEERS LIVING IN ROME: CORRELATION WITH HOMOCYSTEINE LEVELS**

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**Background.** Among various cardiovascular risk factors, hyperhomocysteinemia (HHcy) has reemerged as an important one. The raised levels of homocysteine are determined not only by the known enzyme mutations, but also by nutritional deficiencies in B vitamin cofactors (folic acid, vitamin B6 and B12). The importance of determination of these vitamins is suggested, according to the literature data, because these B vitamins and in particular vitamin B6 (pyridoxine/pyridoxal phosphate) are modulators of cardiovascular risk independently of homocysteine. Aim of our study was to establish reference intervals of vitamin B6 among healthy and young volunteers living in Rome, Italy.

**Patients.** We randomly selected 300 subjects (y. 18-60, mean: 35 y.) from a group of 1241 volunteers living in Rome, whose homocysteine has been previously determined.

**Methods.** Blood samples with EDTA and citrate as preservative were obtained from fasting subjects. Total homocysteine was measured by HPLC with fluorescence detector (Araki and Sako), vitamin B6 was determined by HPLC commercial method (Immunodiagnostik).

**Results.** Vitamin B6 and homocysteine concentration are summarized in Table 1.

We found a statistical significant difference between both Hcy and vitamin B6 by sex ( $p=0.002$  and  $0.003$  respectively) and also a statistical significant difference for vitamin B6 in two different age groups (over and under 35 y.,  $p=0.02$ ). **Conclusions.** the obtained results could justify the usual reference intervals of 5-30 ng/mL for vitamin B6 and of 5-15 micromol/l for homocysteine, in the whole population. However our results, on the basis of the different intake of vitamin B6 and the other vitamins, suggest that would be useful establish different reference intervals by sex and age, mostly for vitamin B6, after further observations about the subject lifestyle.

**Table 1.**

Vitamin B6 (nanog/mL)	Males (189)	Females (111)
median	7.75	5.9
intervals	2.0-38.0	1.6-26.1

Homocysteine (micromol/L)	Males (189)	Females (111)
median	9.1	7.2
intervals	4.6-16.6	3.4-17.0

## P007

**DETERMINANTS FOR VITAMIN B6 AND FOLATE LEVELS IN AN ELDERLY POPULATION**

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Prospective and cross-sectional studies have demonstrated that both low dietary intake and low serum levels of folate and/or vitamin B6 significantly account for increased risk of vascular events. Several studies have demonstrated a crucial role for inflammation in the pathogenesis of cardiovascular disease. A link between inflammation and impaired vitamin B status, independently of homocysteine levels, has been documented. It has been also demonstrated that the MTHFR C677T polymorphism influences circulating folate levels, particularly in subjects in whom the folate intake is inadequate.

The aim of the present study was to determine the relationship between vitamin B6/ folate concentrations and the inflammatory markers, nutrient intakes, and MTHFR genotypes in a large elderly population-based study, which sampled people living in two sites in the surrounding of Florence (Italy), the InChianti Study. 586 men and 734 women randomly selected from the inhabitants of two small towns in the surrounding of Florence, Italy. At multivariate linear regression analysis, after adjustment for multiple potential confounders, higher vitamin B6 concentrations were associated with the intake of beta-carotene as well as the alpha-tocopherol serum levels. Lower concentrations of vitamin B6 levels were significantly associated with albumin and higher concentrations of CRP and IL-6r. Higher folate concentrations was directly associated with the intake of vitamin C and with higher circulating levels of alpha-tocopherol and lower circulating levels of gamma-tocopherol. No significant association between folate concentrations and inflammatory markers was observed. The multiple linear regression analysis demonstrated that folate levels were related to MTHFR C677 genotype in subjects with folic acid intake in the lowest tertile (<221.2  $\mu\text{g}/\text{die}$ ).

Our results demonstrate in an elderly population, a strong, independent association between inflammatory markers and vitamin B6 concentrations and identify CRP and the cytokine IL-6R as major determinants involved in this association. Furthermore, we obtained the novel finding of a relationship between vitamin B6, folate concentrations and alpha-tocopherol concentrations.

## P008

**DETERMINATION OF HUMAN PLASMA PYRIDOXAL 5-PHOSPHATE (VITAMIN B6): EVALUATION OF THREE DIFFERENT METHODS**

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Vitamin B6, measured as pyridoxal 5-phosphate (PLP), is a co-enzyme in the trans-sulfuration pathway of homocysteine metabolism. Since depletion of PLP has been suggested as an independent risk factor for cardiovascular disease,

## Homocysteine Metabolism

PLP is frequently measured to guide patient care. Moreover vitamin B6 is significantly diminished in sideroblastic cell anemia, in diabetic neuropathy, in renal insufficiency and deficiency of PLP has also been associated with immunological changes observed in patients infected with HIV.

Few procedures have been developed for direct measurement of PLP and few comparative reports are available. In this study, we compared three assays: A) vitamin B6 HPLC (Immunodiagnostik, Bensheim, Germany); B) vitamin B6 3H-REA (Bühlmann Laboratories AG, Allschwil, Switzerland); and C) a home-made radioenzymatic assay (REA) based on the selective extraction of [3H]tyramine and its subsequent quantification by liquid scintillation counting after the decarboxylation of high-specific-activity [3H]tyrosine by tyrosine apodecarboxylase enzyme. At variance with the home-made method, the Bühlmann assay does not include a deproteinization step. Intra- and inter-assay imprecision (CV) of the three methods ranged from 4.6% to 9.6%. The study was performed on plasma samples collected in sodium citrate from 99 donors (age range: 30-60 yr), including healthy subjects (n=40) and patients with (n=15) or without (n=44) vitamin B6 supplementation.

Medians and (ranges) were: 34.5 (4.0-415), 37.9 (1.0-508) and 31.0 (1.0-660) nmol/L, for the HPLC, Bühlmann and home-made procedures, respectively. PLP levels were similar with the two commercial procedures and significantly higher ( $p < 0.0001$ ) than those recorded with the home-made method. Correlation coefficients between methods ranged from 0.948 to 0.979. Agreement among the three methods was evaluated in samples from subjects not receiving vitamin B6 supplementation (n=84). Limits of agreement ranged from -80% to 350%.

Thus, despite comparable average values, comparability of PLP levels with the different assays was very poor. Standardization of PLP assays is urgently needed.

### P009

#### EVALUATION OF A NEW ENZYMIC METHOD FOR HOMOCYSTEINE MEASUREMENT: COMPARISON WITH HPLC AND IMMUNOENZYMATIC METHODS

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**Background.** Homocysteine, a non-protein-forming sulphhydryl amino acid, has been the subject of a large number of studies because its strong association with all forms of cardiovascular diseases. The introduction of homocysteine as a risk factor of cardiovascular disease, but also as a marker of nutritional deficient status is now more widespread after the introduction of new commercially available immunoassays that permit a simpler and more rapid measurement of homocysteine. However, the need of special equipment and very expensive reagents for the immunoenzymatic methods has under mandatory the advent of simple new enzymatic fully automated methods for homocysteine determination, also in order to lower the costs of analysis.

**Methods.** In this paper we compare the data obtained with a new enzymatic colorimetric method for homocysteine assay (Carolina Liquid Chemistries), with those obtained with an immunoenzymatic method (Abbott AxSYM immunoassay), in use in our clinical chemistry laboratory, and with those

obtained with a HPLC reference method.

**Results.** The results obtained clearly indicate that the enzymatic method has a good correlation with both the HPLC and AxSYM methods up to homocysteine values lower than 20  $\mu$ mol/L, while the correlation in the whole interval is less significant ( $R^2=0.75$  and  $0.76$  for HPLC and for AxSYM immunoassay methods respectively).

The difference plots analysis shows that the enzymatic method overestimates the homocysteine values mostly in the range over 20  $\mu$ mol/L.

**Conclusions.** This method, although showing a good linearity in the range studied (3-90  $\mu$ mol/L), needs to be improved. We suggest that the actual concentrations of the enzymes used, cystathionine- $\beta$ -lyase activity, could be the rate limiting step; therefore, the formation of cystathionine concentration higher than the theoretical due to the real serum concentration of homocysteine, induces the production of higher amounts of pyruvate and therefore higher rates of NADH oxidation. According to our hypothesis, we suggest to increase the concentration of the cystathionine- $\beta$ -lyase that seems to be the rate limiting step.

### P010

#### FOLATE VITAMER DISTRIBUTION IN WHOLE BLOOD: MEASUREMENT BY HPLC-TANDEM MASS SPECTROMETRY AND ASSESSMENT OF NORMAL VALUES AND DETERMINANTS IN HEALTHY VOLUNTEERS

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**Background.** Different folate vitamers appear during intracellular folate metabolism. Previous studies have identified the MTHFR 677C>T polymorphism as a determinant of erythrocyte folate viter distribution.

**Aims.** To develop a HPLC-tandem mass spectrometry method for measurement of whole-blood folate vitamers and to identify determinants of whole-blood folate viter distribution.

**Methods.** Folates in whole blood lysates were deconjugated in ascorbic acid solutions and, after deproteinization, purified using folate binding protein affinity columns, and concentrated by SPE and evaporation. After LC separation on a C18 column, folates were detected using positive electrospray ionization MS/MS under multiple reaction monitoring conditions. Whole-blood samples of 109 healthy volunteers (52M/57F) were analysed.

**Results.** Reduced folates, except 5-methyltetrahydrofolate (5MTHF), converted to 5-methenylTHF during the assay. Limits of quantification for 5-MTHF and 5,10-methenylTHF were 0.4 nmol/L. Intra-assay and inter-assay CVs for 5-MTHF were 1.2% and 2.8%, respectively. Intra-assay and inter-assay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively. Storage of whole-blood samples in ascorbic acid at low pH resulted in 53-90% loss of the non-methylTHF fraction. In whole-blood samples of healthy subjects, the non-methyl folate/total folate fraction (median, range) was 0.5% (0.0-11.9%) in MTHFR 677CC subjects, 0.9% (0.0-13.8%) in 677CT subjects, and 29.5% (5.5-71.9%) in 677TT subjects ( $p < 0.001$ ). In addition to the MTHFR 677C>T polymorphism, the degree of non-methylfolate accumulation was positively correlated with total folate status, but only in 677CC and CT subjects ( $p < 0.01$ ). Non-methylfolate accumulation did not correlate with the MTHFR 1298A>C polymorphism or with riboflavin status.

**Conclusions.** Whole-blood folate viter distribution can be

accurately measured by HPLC-tandem mass spectrometry. The MTHFR 677CT genotype represents an intermediate phenotype with respect to intracellular non-methylfolate accumulation. Total folate status influences non-methylfolate accumulation.

#### P011

##### SEQUENCE VARIATIONS IN THE THYMIDYLATE SYNTHASE GENE: FREQUENCY AND POSSIBLE INFLUENCE ON HOMOCYSTEINE AND FOLATE METABOLISM

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**Background.** Sequence variations in the thymidylate synthase gene (TYMS) may influence homocysteine and folate metabolism and thus cardiovascular risk. For the maintenance of the deoxythymidine monophosphate (dTMP) pool, thymidylate synthase utilizes 5,10-methylenetetrahydrofolate as a cofactor for the conversion of deoxyuridine monophosphate (dUMP) to dTMP. In addition to a common tandem repeat in the 5' untranslated region, a SNP in this tandem repeat and a 6 bp deletion in the 3' untranslated region, four sequence variations are reported in the coding sequence of this gene. All of these were found in single studies in a limited number of subjects. Epidemiological studies and frequency data are not available yet.

**Aims.** In order to find additional sequence variations which might be associated with homocysteine and folate metabolism, we screened the seven exons of the TYMS in 53 subjects with homocysteine above the 90 percentile and below the 10. percentile selected from a cohort of healthy volunteers.

**Methods.** For mutation screening we used DHPLC, which is a sensitive and straight forward method for the detection of DNA sequence variations in amplicons up to 1000 bp. Results: So far, we did not identify any unknown SNP in the coding sequence of the TYMS gene in our ongoing study. Furthermore in our subjects we did not detect any of the four exonic sequence variations being reported for this gene. **Conclusions.** We suggest that the SNPs described for the TYMS gene are too rare in a middle European population as to serve as reliable predictive markers for homocysteine and folate concentration, respectively.

#### P012

##### POLYMORPHISMS IN GENES INVOLVED IN HOMOCYSTEINE METABOLISM IN THE INCHIANTI STUDY

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Total blood levels of homocysteine (tHcy) have been shown to depend on both environmental and genetic factors, and to be associated with the risk of developing atherosclerosis with its complications of coronary heart disease and stroke. Hyperhomocysteinemia may be associated with the presence of polymorphisms in genes codifying for enzymes involved in the Hcy metabolism. Aim of this study was to evaluate in the InCHIANTI Study, a prospective population-based Italian study of risk factors for disability in late life: 1) total homocysteine, folic acid, vitamin B12 and

B6 levels and 2) the association between homocysteine levels and the genetic polymorphisms of methionine metabolism enzyme: C677T and A1298C of 5, 10-methylenetetrahydrofolate reductase (MTHFR), and A2756G of methionine synthase (MTR) gene polymorphisms. We studied 586 men (78% aged, >65 years) and 734 women (81% aged, >65 years) randomly selected from the people living near Florence. Homocysteine plasma levels were significantly ( $p < 0.0001$ ) higher in men than in women and significantly correlated with increasing age ( $r = 0.46$ ;  $p < 0.0001$ ). Serum folic acid, vitamin B6 and B12 were 6.3 (0.7-45.3) nmol/L, 25.3 (0.4-689.2) nmol/L and 285 (25.2-1488.1) pmol/L respectively. A significant genotype-phenotype association ( $p < 0.01$ ) between homocysteine levels and C677T MTHFR polymorphisms was observed; no significant association with A1298C MTHFR or A2756G MTR polymorphisms was found. Furthermore, in subjects with folic acid <5nmol/L we observed a significant genotype-phenotype association ( $p < 0.001$ ) between homocysteine plasma levels and C677T MTHFR polymorphism, but not in those with folic acid <5nmol/L. Among all the possible combinations of the 3 analyzed polymorphisms, the more frequent combined genotypes resulted 677CT/1298AC/2756AA (28.6%), 677TT/1298AA/2756AA (19.6%) and 677CT/1298AA/2756AA (16.7%). Homocysteine levels were significantly ( $p < 0.01$ ) higher in combination 677TT/1298AA/2756AA, 16.6 (15.8-17.5) than in all the other possible combinations. These data demonstrate that homocysteine levels were influenced by C677T MTHFR polymorphism, but not A1298C MTHFR or A2756G MTR polymorphisms, in subjects with reduced folic acid levels.

#### P013

##### NOVEL MUTATIONS IN THE 5'-UTR OF THE FOLR1 GENE

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**Background.** Folate receptor-alpha mediates the influx of folate to cells. There are no mutations in the exons of its gene, FOLR1.

**Aims.** Our aim was to search for mutations in the 5'-UTR of FOLR1.

**Methods.** We used SSCP and DNA sequencing for screening between nt -116 and nt +207 and between nt -1110 and nt -425, in 92 patients with hyperhomocysteinemia. We have also screened 692 healthy school children (grade 3 or 9), so far between nt -116 and nt +207.

**Results.** In the 92 subjects with hyperhomocysteinemia, we found two patients with a duplication of an A (c.-69dupA), previously discovered by us. Two patients had a novel c.-1043C>T SNP. One patient had two SNPs very close to each other, c.-856C>T and c.-921C>T. In the 692 school children, there were five who had the 25 bp deletion, previously described by us. Three children had a novel C>T SNP (c.-18C>T). In summary, we have described 6 novel FOLR1 mutations. Among 92 subjects with hyperhomocysteinemia, 5.4% had a FOLR1 mutation, among school children 1.2% had a FOLR1 mutation (between nt -116 and nt +207). The impact of these mutations on Hcy levels must be adjusted for each subject's MTHFR 677C>T status.

**Conclusions.** We conclude that the 5'-UTR of FOLR1 is somewhat of a mutational hotspot.

## P014

**THE MOLECULAR BASIS OF DEFECTIVE REGULATION OF CYSTATHIONINE BETA-SYNTHASE BY S-ADENOSYLMETHIONINE**

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**Background.** Homocystinuria due to cystathionine beta-synthase (CBS) deficiency is an inborn error of methionine metabolism, and is inherited as an autosomal recessive trait. S-adenosylmethionine (AdoMet), an intermediate in the conversion of methionine to homocysteine, is the methyl donor in various transmethylation reactions. In addition, AdoMet is an important allosteric regulator of the homocysteine flux through either the transsulfuration or remethylation pathway; it activates CBS activity and decreases the formation of 5-methyltetrahydrofolate by inhibition of methylenetetrahydrofolate reductase (MTHFR). In the past, a D444N mutation in the regulatory domain of CBS was identified, that interfered in the regulation by AdoMet (Kluijtmans *et al.* J.Clin.Invest 1996;98:285-289). Ever since, we have included an AdoMet-dependent assay in the enzymatic diagnostics of homocystinuria. About 15% of the patients with CBS dysfunction have a defective regulation of CBS. **Aims.** In this study we are trying to elucidate the mutations in the CBS gene responsible for this impaired AdoMet-stimulation.

**Methods.** Up till now, we have studied the AdoMet regulation of CBS activity in cultured fibroblasts and identified an impaired AdoMet response in 15 patients with a decreased, but not deficient CBS activity and in 4 patients with a normal CBS activity. The coding region including the exon-intron boundaries was screened for mutations by sequencing analysis.

**Results.** Sequencing analysis of the CBS genes in these patients (~50% analyzed so far) revealed 3 missense mutations and 1 deletion. These mutations will be cloned into E.coli to confirm their CBS dysregulation by AdoMet.

**Conclusions.** We found 4 mutations which might be involved in defective regulation of CBS by AdoMet. The identification of these mutations will provide more information about the mechanism by which AdoMet regulates CBS activity.

## P015

**EFFECT OF GENETIC POLYMORPHISM ON HOMOCYSTEINE AND CYSTEINE LEVELS IN INDIAN POPULATION**Kumar J,<sup>1</sup> Sharma P,<sup>1</sup> Das SK,<sup>1</sup> Karthikeyan G,<sup>2</sup> Lakshmy R,<sup>2</sup> Sengupta S<sup>1</sup><sup>1</sup>*Institute of Genomics and Integrative Biology, Mall Road, Delhi;*<sup>2</sup>*All Indian Institute of Medical Sciences, Ansari Nagar, New Delhi, India*

**Background.** Hyperhomocysteinemia has been associated with various complex disorders. Homocysteine levels are elevated either by environmental (including diet) and/or genetic factors. A majority of Indian population is deficient in folate and vitamin B12, the two critical nutritional factors, deficiency of which result in hyperhomocysteinemia. Thus, polymorphisms in the genes responsible for the metabolism of homocysteine can be perceived to have a greater impact vis-à-vis hyperhomocysteinemia in Indian population.

**Aims.** We wanted to evaluate the effect of genetic polymorphism on homocysteine and cysteine levels in Indian population.

**Methods.** 100 individuals with varying plasma homocysteine and cysteine concentrations (determined using HPLC-FD) were genotyped for 20 non-synonymous single nucleotide polymorphism (ns SNP) in 8 genes, directly or indirectly involved in homocysteine metabolism.

**Results.** Only 9 of these 20 nsSNPs showed a minor allele frequency (MAF) of >5%. The MAF of 5 nsSNPs were similar to that reported in Caucasians. However, MAF of MTHFR C665T was lower (0.15) in Indian population as compared to Caucasians (0.35-0.4). Similarly, the MAF of MTRR A1049G was found to be lower while that of Choline dehydrogenase (CHDH) T233G and A119C were higher in Indian population. Furthermore, CHDH A119C polymorphism showed significant genotypic association with homocysteine levels ( $p=0.001$ ) under additive model. QTL association analysis indicates that C allele significantly ( $p=0.01$ ) tends to increase the plasma homocysteine level. Interestingly, CTH G1208T ( $p=0.033$ ), MTHFR C665T ( $p=0.019$ ) and G1781A ( $p=0.024$ ) were significantly associated with plasma cysteine concentration.

**Conclusions.** We conclude that the MAF of some of the common SNPs in Indian population vary considerably from other populations. Furthermore, these polymorphisms correlate with plasma homocysteine and/or cysteine concentrations. The levels of folate and vitamin B12 are currently being determined to evaluate the effect of diet and genotype-nutrient interaction on the levels of homocysteine and cysteine.

## P016

**MATERNAL POLYMORPHISMS OF FOLATE/HOMOCYSTEINE PATHWAY, GENE-GENE INTERACTIONS AND RISK OF DOWN SYNDROME**Scala I,<sup>1</sup> Granese B,<sup>1</sup> Sellitto M,<sup>1</sup> Salomè S,<sup>1</sup> Sammartino A,<sup>2</sup> Pepe A,<sup>1</sup> Fratellanza G,<sup>3</sup> Sebastio G,<sup>1</sup> Mastroiacovo P,<sup>4</sup> Andria G<sup>1</sup><sup>1</sup>*Dept. of Pediatrics, Federico II University, Naples;* <sup>2</sup>*Dept. of Gynecology and Obstetrics, Federico II University, Naples;* <sup>3</sup>*Dept. of Transfusion Medicine and Immunohaematology;* <sup>4</sup>*International Centre on Birth Defects, Rome, Italy*

**Background.** Down syndrome (DS) is caused by trisomy of chromosome 21. Advanced maternal age is the only well documented risk factor for chromosomal nondisjunction, although the underlying mechanisms remain elusive. The molecular bases of DS occurring in young women offspring are also unknown. Genetic polymorphisms of folate pathway were studied in DS mothers with discordant results. We present a case-control study in a homogeneous population on seven polymorphic variants [methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, cystathionine-beta-synthase (CBS) 844ins68, methionine synthase reductase (MTRR) A66G, methionine synthase (MTR) A2756G, reduced folate carrier1 (RFC1) A80G, methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthase (MTHFD1) G1958A] as risk factors for DS. Gene-gene interactions and association with maternal age at conception were also evaluated.

**Aims.** To analyse the contribution of polymorphisms of folate pathway to increased risk of DS. **Methods.** DNAs from 83 DS-mothers and from 263 control-mothers from Campania, Italy, were amplified through PCR reaction and analysed by specific restriction enzymes.

**Results.** Increased risk of DS was associated with the MTHFR 1298C allele (OR 1.56; 95%CI 1.04-2.33) and 1298CC genotype (OR 2.59; 95%CI 1.11-5.99), the RFC1 80G allele (OR 1.52; 95%CI 1.02-2.28) and the RFC1 80GG

genotype (OR 1.96; 95%CI 0.91-4.21). Significant associations were found between the MTHFR 1298C (OR 1.95; 95%CI 1.07-3.58), MTR2756G (OR 3.82; 95%CI 1.38-10.71) and RFC180G (OR 2.09; 95%CI 1.13-3.87) alleles respectively and maternal age over 30 years. The MTHFR 677TT genotype was associated with maternal age <30 years (OR 5.75; 95%CI 1.28-29.51). A positive interaction was found between the MTHFR 677TT and the 1298CC/CA genotypes, while a protective effect was shown when the MTHFR 677TT and MTR GG/GA genotypes were present in the same woman. **Conclusions.** Data reported in this study support the role of polymorphisms of folate/homocysteine pathway as risk factors for chromosome 21 nondisjunction.

### P017

#### HPLC METHOD TESTING FOR DETERMINATION OF BETAINE AND DIMETHYLGLYCINE IN BLOOD AND URINE

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**Introduction.** Betaine can decrease plasma homocysteine by transmethylating it to methionine. The removal of a methyl group from betaine leads to dimethylglycine (DMG). Analysis of betaine and its metabolites in biological materials are not yet routine methods. This study was to clarify if the previously described HPLC method by Laryea *et al.* (1998) can be routinely used for these analyses.

**Methods.** Venous blood and 24-h urine samples were collected from 32 healthy adults before and after betaine intervention (4 g/day, 24 weeks). Plasma was obtained by centrifuging the blood samples. Betaine and DMG were determined as bromophenyl ester derivatives with UV detection (254 nm). The analytical methods were tested for linearity, sensitivity, accuracy and repeatability. The effect of blood and urea matrix for the obtained betaine and DMG results was examined by recovery tests.

**Results.** The method was found to be linear for betaine and DMG within the studied concentration range 5 to 2500 micromol/L. The mean recoveries of betaine and DMG added to blood were 105% and 93%, respectively. In urea matrix the recoveries were for betaine 103% and for DMG 95%. The uncertainty of the betaine results was estimated by day-to-day tests (2,7xRSD, n=30). Both in urea and blood matrix found to be +13%. The uncertainty of the DMG results was +171% in blood matrix and +20% in urea matrix. Betaine concentrations in plasma (from 25,6±9,6 to 187,3±107,8 μmol/L) and urine (from 82,0±52,6 to 954,1±1186,8 μmol/L) as well as betaine excretion (from 115,8±56,6 to 1358,6±1393,8 micromol/day) increased during betaine intervention.

**Conclusions.** The studied HPLC method can be used for quantitative analysis of betaine in blood and urea samples. However the sample matrix disturbs DMG determination especially in blood.

### P018

#### PHYSICAL ACTIVITY AFFECTS ALL FORMS OF PLASMA LEVELS OF HOMOCYSTEINE

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Regular physical activity is considered to reduce plasma homocysteine (Hcy), but higher Hcy levels are consistently found after acute exercise. Furthermore, production of reactive oxygen species during exercise could change the thiol redox status and explain the underlying mechanism.

**Aims.** We investigate the influence of the extension of previous training period in the increase of Hcy after acute exercise and the adaptation of the different plasma forms to the sustained oxidative stress.

**Methods.** An elite cycling team was kept throughout a complete sporting season. Participants, aged 20.9±1.0 years, undertook a specific cycling test at 100% VO<sub>2</sub>max in January (n=15, lower training load), March (n=10) and June (n=8, higher training load) and acidic citrate (Stabilyte) samples were obtained before and 30 minutes after completing the test. Likewise, blood sampling took place before and 30 minutes after a summer race, July-August (n=12). Using ultrafiltration as the separation method for the protein bound forms, plasma levels of reduced, free and total aminothiols were measured by reversed-phase HPLC after derivatization with SBD-F. Paired t-test was used for comparisons within acute tests and one-way ANOVA between months for differences in increases.

**Results.** Results after physical activity were adjusted for changes in plasma concentration. Acute exercise increased significantly the levels of all forms of Hcy in every group. However, the increases between samples were not significantly different within groups of training. Likewise, decreases in ratios of reduced to total Hcy after exercise were observed in all groups but it was significant just for the June group.

**Conclusions.** The increasing effect of exercise on Hcy involves all forms present in plasma but it is independent of the training level achieved. The oxidative stress of the acute exercise seems to be shown by the relative increase of the oxidized forms but larger experimental groups are needed for demonstration.

Table 1.

	January		March		June		July-August	
	x±sd	p<	x±sd	p<	x±sd	P <	x±sd	p<
Hcy	6.68±1.06		7.38±1.21		6.52±0.98		6.48±1.45	
total	7.87±1.29	0.001	8.34±1.29	0.01	7.11±1.12	0.01	8.01±2.12	0.001
Hcy	1.54±0.20		1.64±0.22		0.87±0.19		1.24±0.39	
free	2.00±0.28	0.001	1.83±0.26	0.05	1.10±0.19	0.01	1.96±0.75	0.001
Hcy	0.15±0.02		0.17±0.03		0.18±0.03		0.16±0.03	
reduced	0.17±0.02	0.001	0.19±0.03	0.01	0.18±0.03	NS	0.18±0.05	0.05

P019

**REGULATION OF THE 5-FORMYLTETRAHYDROFOLATE FUTILE CYCLE AND PURINE BIOSYNTHESIS**

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5-formyltetrahydrofolate (5-formylTHF), which represents about 3-10% of total cellular folates, does not serve as a co-factor in one-carbon metabolism. 5-formylTHF is synthesized from methenyltetrahydrofolate in a reaction catalyzed by serine hydroxymethyltransferase (cSHMT); the enzyme methenyltetrahydrofolate synthetase (MTHFS) catalyzes the reverse reaction which is ATP dependent. Therefore, MTHFS and SHMT constitute a futile cycle that regulates intracellular 5-formylTHF concentrations. 5-formylTHF is a potent inhibitor of several folate-utilizing pathways such as purine biosynthesis and enzymes including SHMT, which also reversibly converts THF and serine to glycine and 5,10-methyleneTHF. This reaction generates most one-carbon units required for purine synthesis (shuttled through THF), thymidylate (5,10-methyleneTHF serves as the carbon donor), and methionine synthesis (after 5,10-methyleneTHF is converted to 5-methylTHF). Because MTHFS is poised to regulate the accumulation of the inhibitor 5-formylTHF, it may regulate folate-mediated one-carbon metabolism and therefore MTHFS is an attractive therapeutic target. A series of 5-formylTHF analogs were synthesized and investigated as potential substrates and inhibitors of MTHFS. The results from these studies indicate that the pABG moiety, but not the glutamate moiety of THF is essential for MTHFS activity, and that polyglutamation beyond triglutamation contributes significantly to MTHFS substrate specificity and inhibitor function. Furthermore, we have determined that 10-formylTHF, which exists in chemical equilibrium with the MTHFS reaction product 5,10-methenylTHF, acts as a highly effective tight-binding inhibitor of MTHFS, thus regulating the 5-formylTHF futile cycle *in vivo*. Results from *in vitro* kinetic studies, molecular modeling studies and cell culture studies indicate that MTHFS regulates purine biosynthesis by 1) metabolizing the inhibitor 5-formylTHF and 2) sequestering the purine synthesis co-factor 10-formyltetrahydrofolate.

P020

**THE MTHFR 1298 A->C POLYMORPHISM. EFFECTS ON SERUM FOLATE AND TOTAL HOMOCYSTEINE**

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**Background.** The MTHFR1298 A->C is a functional polymorphism of the MTHFR enzyme and has been implicated in the etiology of several diseases.

**Aims.** To evaluate the association of MTHFR1298A->C with serum folate and plasma total homocysteine levels.

**Methods.** About 10.000 subjects from the Norwegian Colorectal Cancer Prevention study (NORCCAP) were analysed for polymorphisms by a high-throughput multiplexed assay based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The subjects were 50-64 years old, and recruited from an average-risk Norwegian population. 13 different polymorphisms

related to folate and homocysteine metabolism were assayed including methylenetetrahydrofolate reductase (MTHFR) 677C->T and 1298A->C. Serum total homocysteine was determined by GC-MS, and serum folate by a microbiological assay.

**Results.** Our results confirmed a strong linkage disequilibrium between the MTHFR1298A->C and 677C->T polymorphisms. For subjects with the 677CC genotype, serum total homocysteine increased, and serum folate decreased significantly according to the number of 1298C alleles. The effect on total homocysteine was most pronounced at high serum folate. This is in contrast to the 677C->T polymorphism, which increased homocysteine more in the low quartile than in the high quartile of folate. No interaction was found between the two polymorphisms with respect to homocysteine or folate levels, however, as have been reported previously, subjects with 677CT/1298AC had the second highest homocysteine level and second lowest folate level.

**Conclusions.** The MTHFR 1298A->C polymorphism was significantly related to homocysteine and folate levels (within the 677CC-group). Moreover, the effect of 1298A->C on homocysteine was modified by the folate level.

P021

**TRANSULFURATION RATES IN CRITICALLY ILL SEPTIC CHILDREN**

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**Objective.** To investigate the rates of methionine transulfuration in critically ill septic children, receiving limited parenteral nutritional support.

**Design.** Prospective study.

**Setting.** Multidisciplinary intensive care unit at a children's hospital.

**Patients.** Six septic pediatric patients.

**Interventions.** Septic patients (age, 2 months to 15 yrs) received a 9-hr primed, constant, intravenous tracer infusion of L-[1-13C] methionine and L-[2H2] cysteine, and L-[5,5,5,2H3] leucine. Blood samples were obtained for determination of plasma isotopic enrichment and of 13CO<sub>2</sub> by mass spectrometric techniques. The plasma fluxes of leucine, methionine and cysteine and the rates of methionine transulfuration were determined. Septic patients received limited nutritional support as per routine clinical management.

**Measurements and Main Results.** Plasma methionine, cysteine and leucine fluxes were respectively, 31±3, 59±8 and 186±35 µmol.kg<sup>-1</sup>.h<sup>-1</sup> (mean±SD). The rates of methionine transulfuration were 7.7±1.8 µmol.kg<sup>-1</sup>.h<sup>-1</sup> (mean±SD). Protein balance was negative at -0.76±0.35 g.kg<sup>-1</sup>.d<sup>-1</sup>.

**Conclusions.** Critically ill septic children receiving limited parenteral nutritional support, utilize about 25.5% of plasma methionine flux for oxidative disposal, while 74.5% is utilized for non-oxidative disposal. However, healthy adults in the post-absorptive state utilize about 14.5% and 85.5% for oxidative and non-oxidative disposal, respectively (1). It appears that septic children have a greater utilization of plasma methionine flux for transulfuration rates. It remains to be determined if supply of cysteine decreases the rates of methionine transulfuration in these patients.

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## P022

**HYPERHOMOCYSTEINEMIA INHIBITS POST-INJURY REENDOTHELIALIZATION IN CBS MICE**

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Hyperhomocysteinemia (HHcy) is a risk factor for cardiovascular disease and has been reported to inhibit endothelial cell (EC) growth. The effect and mechanisms by which HHcy regulates EC growth *in vivo* are largely unknown. In this study, we established a mouse carotid artery air-dry endothelium denudation and reendothelialization model, and evaluated the effect of HHcy on post-injury reendothelialization in mice with the gene deletion of cystathionine  $\beta$ -synthase (CBS). Severe HHcy was induced in CBS<sup>-/-</sup> mice with a high methionine diet. Post-injury reendothelialization was impaired, correlating with increased neointima formation in hyperhomocysteinemic mice. To elucidate the underlying mechanism, we examined circulating endothelial progenitor cells (EPC) in hyperhomocysteinemic mice, and studied *in vitro* effect of Hcy on proliferation and migration of human umbilical vein endothelial cells (HUVEC), and on adhesion in mouse EPC and HUVEC. Circulating EPC were slightly decreased in HHcy mice. In addition, Hcy inhibited proliferation and migration of HUVEC, and decreased adhesion of EPC and HUVEC to fibronectin. These data indicate that Hcy inhibits post-injury reendothelialization and leads to intimal hyperplasia. The capacity of Hcy to inhibit proliferation and migration of EC, and adhesion of EC and EPC, may be responsible for impaired reendothelialization and contribute to arteriosclerosis in HHcy.

## P023

**PROSPECTIVE STUDY OF HOMOCYSTEINE METABOLISM IN CARDIOVASCULAR DISEASES IN SOUTHERN FRANCE**

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Moderate hyperhomocysteinemia has been observed in patients with cardiovascular diseases. A prospective study of homocysteine metabolism in 149 patients who had suffered a coronary event or stroke and 111 healthy controls was conducted at the Nice Hospital System. Plasma homocysteine (tHcy) (FPIA), folate, B12 (microbiology) and B6(HPLC) were assayed and three common mutations were sought: the C677T mutation (MTHFR gene), A2756G (MTR gene), and A66G (MTTR gene). tHcy was significantly higher in the patients than in the controls (12.8±6.2/10.0±4.4 μmol/L) and was inversely correlated with folate status in the patients. Vitamin deficiencies were more prevalent in the patients than in the controls; this was especially true for folates (25% versus 15.5%) and pyridoxal phosphate (20.5% versus 8.6%) and, to a lesser degree, vitamin B12 (8.5% of the patients versus 6.9% of the controls). The percentage of homozygotes for the C677T mutation was not significantly higher in the patients than in the controls: 16.1% versus 15.3%. tHcy was higher in the presence of the TT geno-

type; so for the A66G mutation: 24.8%, in patients, not higher than in controls: 23.1%, without influence of the genotypes on tHcy values. The percentage of homozygotes for the A2756G mutation was 2.7% in patients and 3.6% in controls, but tHcy was lower in GG subjects.

*Conclusions.* average tHcy was higher in the patients than in the controls, due in part to the fact that a high percentage of the patients had a vitamin deficiency, primarily in folate and vitamin B6, that contributes to hyperhomocysteinemia, often associated with a genetic profile, a genotype GG (C677T) and AA (A2756G). Such a vitamin deficiency was observed in a high number of patients, even though in the south of France is characterized by a diet rich in folate.

## P024

**EFFECT OF LIPID-LOWERING DRUGS THERAPY COMBINED WITH FOLATE SUPPLEMENTATION ON THROMBIN GENERATION IN HIGH CARDIOVASCULAR RISK SUBJECTS**

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*Background.* An increasing body of evidence supports the view that statins and fibrates have antithrombotic effects of potential clinical importance. Homocysteine (tHcy) has been shown to enhance platelet activation and increase prothrombotic potential that predispose to thrombotic complications of atherosclerosis and venous thrombosis.

*Aims.* To determine whether treatment with hypolipemic drugs combined with folic acid supplementation may suppress thrombin generation.

*Methods.* Forty-two patients (29 M, 13 F; mean age 54.4 years) with high risk of coronary artery disease and LDL-cholesterol level >130 mg/dL were studied. Blood was sampled four times: at baseline, after a month-long therapy with either simvastatin 40 mg daily (Zocor, Merck Sharp Dohme, n=20) or micronized fenofibrate 160 mg daily (Lipanthyl Supra, Fournier Laboratoires, n=22), and also after 2 months of additional supplementation with folic acid 0.4 mg daily (Folik, Polfa Grodzisk). For the following 4 months all patients took 40 mg simvastatin and 0.4 mg of folic acid daily. In plasma and in the blood collected from skin incisions thrombin-antithrombin complexes (TAT), tHcy and folate levels in plasma were evaluated.

*Results.* Plasma TAT levels decreased significantly and comparably after a month-long therapy with both hypolipemic drugs and remained unchanged during the combined therapy with folate. Thrombin generation in the bleeding-time blood was suppressed in propagation phase, starting from the 120th second already after one month in both groups. Additional significant attenuation of thrombin generation after 6 months of combined therapy was observed. Total thrombin generated expressed as the area under the curve was decreased by 40% ( $p=0.0009$ ). A fall in tHcy (from 12.92±4.64 micromol/L to 9.74±2.45 micromol/L) that correlated with an increase in plasma folate ( $r=0.41$ ;  $p<0.05$ ) was also observed.

*Conclusions.* Combined therapy (statin and folate) may prove of additional benefit on prothrombotic state in patients with hyperhomocysteinemia.

## P025

**ADEQUATE DOSAGE OF ORAL CYANOCOBALAMIN IN VITAMIN B12 DEFICIENCY AND HOMOCYSTEINE LOWERING**Nilsson M,<sup>1</sup> Norberg B<sup>2</sup><sup>1</sup>Departments of Family Medicine; <sup>2</sup>Internal Medicine, University Hospital of Northern Sweden, Sweden

**Background.** In case homocysteine lowering should be generally accepted for large patient populations, it is essential to evaluate the documentation for a safe and reliable dose of cobalamin in treating elderly people over decades. The reason is that many elderly patients will proceed to severe B12 malabsorption during this stage of life.

**Aims.** To review the historical documentation of oral cyanocobalamin, 0.5 mg daily, and 1 mg daily, in deficiency treatment (1,2).

**Methods.** In the first study of the Berlin group (1), all patients (n=64) except 5-12 were treated with oral cyanocobalamin, 0.5 mg daily. In the second study (2), 47 patients out of 64 were treated with oral cobalamin, 1 mg daily.

**Results.** In the first study (1), the median value of serum cobalamin was 205 pmol/L, interquartile range 170-280, range 95-395. In the second study (2), the median value of serum cobalamin was 470 pmol/L, interquartile range 280-580, range 130-675.

**Conclusions.** Oral cyanocobalamin, 1 mg daily, is documented as a safe and reliable long-term prophylaxis and treatment of vitamin B12 deficiency (1-4). Hitherto, lower cobalamin doses are proven unsafe (1). Thus, it is suggested that cyanocobalamin, 1 mg daily, is chosen as folate adjunct in future trials of homocysteine lowering.

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## P026

**THE WHOLE BLOOD COAGULATION PROFILE IN HYPERHOMOCYSTEINEMIC PATIENTS WITH PRIOR THROMBOSIS IS CHANGED BY FOLIC ACID TREATMENT AS EVALUATED BY ROTEG THROMBELASTOGRAPHY**Ebbesen LS,<sup>1,2</sup> Christiansen K,<sup>2</sup> Ingerslev J<sup>2</sup><sup>1</sup>Clinical Institute, University Hospital of Aarhus, Denmark; <sup>2</sup>Centre for Hemophilia and Thrombosis, Department of Clinical Biochemistry, University Hospital of Aarhus, Denmark

Hyperhomocysteinemia (HH) is an independent risk factor for thrombosis although the exact pathogenesis is still not elucidated. Previously, an animal model of HH was used to demonstrate that the whole blood coagulation profile (WBCP) in HH is changed in a thrombogenic direction characterized by a prolongation of the initiation phase, an increase of the velocity and of the maximal clot firmness. We hypothesized that the WBCP were changed similarly in patients suffering HH and that the WBCP could be *normalized* by folic acid treatment as demonstrated previously in our animal study.

**Materials and Methods.** Patients (N=23), who previously had a thrombosis, were admitted to our centre and were diagnosed with HH and were included in this study. All patients were treated with folic acid 5 mg/d for 2 weeks thereafter 5 mg/w. Pre- and post treatment tHcy and WBCP

were recorded. Human recombinant TF was added to citrated whole blood, and coagulation was initiated by Ca<sup>++</sup>-addition. The WBCP were recorded by the roTEG Thrombelastograph (Pentapharm, Munich, Germany).

**Results.** Pre-treatment mean tHcy was 16.9 µmol/L and post-treatment mean tHcy was 9.9 µmol/L, *p*<0.001. The initiation phase of coagulation was reduced by treatment; i.e. the mean pre-CT (clotting time) was 291.1 s and the mean post-CT was 262.5 s, *p*<0.05. Furthermore, the mean pre-Tmax (time to max velocity) was 467.5 s and the mean post-Tmax was 434.5 s, *p*<0.05. No change in velocity (0.185 and 0.199 mm/s) and maximal clot firmness (62.7 and 63.0 mm) were detected.

**Conclusions.** The WBCP in HH patients is changed similarly to what has previously been detected in HH animals; a prolongation of the initiation phase during HH and the CT is reduced by treatment with folic acid. However, no change in velocity and maximal clot firmness were detected, which might be due to present effective antiplatelet-aggregation therapy.

## P027

**HIGH-DOSE FOLATE THERAPY REDUCES HOMOCYSTEINE PLASMA LEVELS IN SUBJECTS WITH EARLY CARDIOVASCULAR FAMILIARITY AND/OR WITH CARDIOVASCULAR EVENTS**Assanelli D,<sup>1</sup> Brentana L,<sup>2</sup> Grassi M,<sup>3</sup> Boldini A,<sup>2</sup> Salvadori G,<sup>1</sup> Albertini F,<sup>2</sup> Maccalli P,<sup>2</sup> Archetti S,<sup>4</sup> Ruggeri G,<sup>4</sup> Negrini R<sup>4</sup><sup>1</sup>Professor, Chair of Internal Medicine, University of Brescia; <sup>2</sup>Medical Doctor, Chair of Cardiology, University of Brescia; <sup>3</sup>Professor, Department of Health Sciences, Section of Medical Statistics & Epidemiology, University of Pavia; <sup>4</sup>Medical Doctor, III Laboratory, Spedali Civili, Brescia, Italy

**Background.** Homocysteine (Hcy) is an important and independent cardiovascular risk factor. Its plasma levels depend on daily vitamin intake and on the presence of genetic mutations on 5,10-methyltetrahydrofolate reductase (MTHFR) and cystathionine beta-synthase (CBS) genes.

**Method.** The main aim of this paper was to evaluate the effect of high and prolonged folate administration in order to reduce plasma Hcy levels in subjects with early cardiovascular familiarity and/or with premature cardiovascular events. The secondary aim was to evaluate the pre-treatment characteristics able to modulate the response to therapy.

**Design.** We considered 89 subjects with Hcy >10 µmol/L, divided into three groups based on the presence of familiarity and vascular events: 31 subjects belonged to the YES-Familiarity and NO-Vascular-Events group, 25 to the NO-Familiarity and YES-Vascular-Events group, and 33 to the YES-Familiarity and YES-Vascular-Events group. We administered 5-methyltetrahydrofolate (15 mg/day) for 30 days, followed by 15 mg on alternate days for 60 days. **Results.** Pre-treatment Hcy levels, sex and alcohol consumption had statistically different effects; therapy led to a 25-61.5% decrease in Hcy level, depending on the pre-treatment Hcy level. The treatment gave a similar and persistent reduction in both females and males (47% versus 47.3%). Subjects who drank two glasses of wine a day had a more intense (= more marked?) response to therapy compared to abstainers (52.8% versus 41.7% reduction in post-treatment) and a lower rebound in the wash-out period (6.5% versus 18.2%).

**Conclusions.** High 5-methyltetrahydrofolate administration significantly reduces homocysteine plasma levels.

## P028

**HYPERHOMOCYSTEINEMIA AND MORTALITY AFTER CORONARY ARTERY BYPASS GRAFTING**

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**Background.** The independent prognostic impact of hyperhomocysteinemia (HHCY) in patients with chronic ischemic heart disease is still controversial.

**Aims.** To evaluate the relationship between HHCY and mortality in patients after coronary artery bypass grafting (CABG) surgery.

**Methods.** We prospectively followed 353 patients (mean age 60.1±9.0; 83.3% males) who underwent elective CABG between May 1996 and May 1999. Before CABG, risk factors for cardiovascular disease, including homocysteine, were evaluated.

**Results.** after a median follow-up of 58 months, 36 patients (10.1%) had died, 28 because of cardiovascular events. Baseline homocysteine levels >90th of distribution (i.e. >25.2 micromol/L) were independently associated to total mortality: OR = 2.5, 95% CIs 1.06-6.01,  $p=0.035$ , after adjustment for age, sex, baseline renal function (glomerular filtration rate estimated by the MDRD equation), and high-sensitivity C Reactive Protein, by logistic regression. HHCY was also independently associated with mortality due to cardiovascular events, adjusted as above including the presence or absence of myocardial infarction prior to CABG (OR =2.7, 95% CIs 1.02-7.17,  $p=0.045$ ).

**Conclusions.** HHCY is independently associated to total and cardiovascular mortality after CABG.

## P029

**EFFECT OF MTHFR GENE POLYMORPHISMS (C677T & A1298C) ON HOMOCYSTEINE LEVELS IN ACUTE MYOCARDIAL INFARCTION PATIENTS AMONG TAMILIANS**

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Hyperhomocysteinemia, due to a combination of genetic and environmental factors, is considered to be a risk factor for coronary artery diseases. 5,10-methylenetetrahydrofolate reductase (MTHFR) is a crucial enzyme in homocysteine metabolism. Mutations in the MTHFR gene have been reported to reduce enzyme activity and are associated with hyperhomocysteinemia. The distribution of genotypes and allele frequencies for MTHFR (C677T and A1298C) gene and corresponding homocysteine concentrations were determined in 100 angiographically proven acute myocardial infarction (AMI) patients and 70 healthy individuals. Homocysteine concentrations were determined using automated chemiluminescence assay and MTHFR genotyping was performed using polymerase chain reaction followed by restriction analysis. This study shows that there is significant elevation of plasma homocysteine concentrations in AMI patients than that of the control group ( $p<0.001$ ). The homocysteine concentrations in patients and controls were 29.74±5.43  $\mu\text{mol/L}$  and 14.91±0.14  $\mu\text{mol/L}$  respectively. The effect of MTHFR C677T mutant genotypes on homocysteine level was more pronounced in cases than in the con-

trols. The homocysteine concentration in patients with mutant genotypes (677TT and CT) is significantly higher in patients when compared to the normal genotype (677CC). Irrespective of the normal or mutant genotype for MTHFR A1298C, the homocysteine levels are markedly increased in patients than in the controls. Our results suggest that, Hyperhomocysteinemia is more prevalent in AMI patients among Tamilians, the MTHFR A1298C mutation has less impact on homocysteine levels and MTHFR C677T may be associated with AMI.

## P030

**HOMOCYSTEINE AND CEREBROVASCULAR ACCIDENT. IS THERE ANY RELATION?**

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High levels of tHCy in plasma, have been suggested by many authors, that are related with a high risk of coronary disease, cerebrovascular accident and phlebothrombosis. **Aims.** The aim of our study was to evaluate the levels of tHCy in relatively young patients, who suffered a transient ischemic attack (TIA) or stroke.

**Materials and Methods.** 145 patients were examined (73 men and 72 women), that have been admitted in the hospital for a cerebrovascular accident (CVA). In case of TIA, the diagnosis was confirmed by the typical neurological symptoms, otherwise it was confirmed by brain CT-scan and/or MRI. We excluded all patients with other predisposing factors, like hypertension, hyperlipidemia and diabetes mellitus. A full cardiologic check up (with U/S and ECG) was normal and also the examination of carotids and vertebralbasal system (with Triplex) was negative. Mean age was 49,4 years old for men (range of age 7-66) and 48,7 for women (range of age 16-69). 250 apparently healthy volunteers blood donors were used as a comparison group. They were all men of mean age years old (range of age 23-55). tHCy was measured with Elisa method (Abbot-AxSYM).

**Results.** Patients and healthy volunteers were divided in two categories: those who had tHCy  $<15 \mu\text{mol/L}$  and those with tHCy  $>15 \mu\text{mol/L}$ .

Table 1.

	WOMEN	MEN BLOOD DONORS	
tHCy>15 $\mu\text{mol/L}$ :	11(15,3%)	16(21,9%)	53(18,4%)
	WOMEN	MEN	MEN BLOOD DONORS
tHCy mean level:	8,9±2,82 $\mu\text{mol/L}$	10,8±2,89 $\mu\text{mol/L}$	12,9±1,9 $\mu\text{mol/L}$

**Conclusions.** No important difference was found in the homocysteine plasma levels between patients and healthy ones. There is a crucial need for full understanding of the possible relevance of hyperhomocysteinemia with vascular disease. More studies are necessary, so that our therapeutic approach could be done in time and is beneficial as well.

## P031

**LIPID LOWERING DRUGS IN PATIENTS WITH HYPERHOMOCYSTEINEMIA**

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The aim of our study was to evaluate the effect of lipid-lowering drugs on plasma total homocysteine (tHCy) levels in patients with combined hyperlipoproteinemia and hyper-

## Homocysteine Metabolism

homocysteinemia ( $>14 \mu\text{mol/L}$ ) operated on for arterial occlusive disease. Our study consisted of 40 participants (27 M, 13 F). Patients were taken off lipid-lowering medication and vitamin supplements for the period of 6 weeks before the study. The treatment began with simvastatin 20 mg/day in the first phase of 2 months. In patients ( $n=18$ ) with inadequate decrease of triglycerides, combination therapy with micronized fenofibrate 160 mg/day was used for the period of 2 months. Next treatment (2 months) consisted of either simvastatin plus vitamins or simvastatin - fenofibrate plus vitamins. The vitamin supplement contained 1 mg folic acid, 20  $\mu\text{g}$  vitamin B12 and 5 mg vitamin B6. Before and after treatment periods, fasting venous blood samples were taken for measurement of tHcy, vitamins (folate, B6, B12) and lipid parameters. MTHFR C677T and A1298C mutations were determined. In the group of patients with hyperhomocysteinemia (tHcy:  $22.88 \pm 6.67 \mu\text{mol/L}$ ) the frequency of the homozygous /TT/ genotype of MTHFR C677T was 30%, A1298C polymorphism - 10%, heterozygotes for both polymorphisms - 15%. We did not find significant association between simvastatin treatment and tHcy level (-4.6%, n.s.). Mean tHcy ( $25.33 \pm 6.34 \mu\text{mol/L}$ ) in statin - fibrate group was significantly higher (+22.9%) than in statin group ( $21.72 \pm 5.94 \mu\text{mol/L}$ ,  $p < 0.05$ ). Changes in tHcy levels on lipid-lowering therapy were not associated with changes in plasma levels of vitamins. Co-administration of multivitamin decreased tHcy to  $13.25 \pm 1.54 \mu\text{mol/L}$  in statin group (-36.8%,  $p < 0.001$ ) and to  $15.10 \pm 2.33 \mu\text{mol/L}$  in statin - fibrate group (-38.3%,  $p < 0.001$ ). Statin - fibrate combination can increase tHcy concentrations in patients with hyperhomocysteinemia. Vitamin supplementation could reduce the elevated tHcy levels in patients on statin or statin - fibrate therapy.

### P032

#### HOMOCYSTEINE AS A MAJOR CAUSATIVE FACTOR FOR CVD AND ALZHEIMER'S DISEASE PART 1: A MECHANISTICAL PROPOSITION FOR CARDIOVASCULAR DISEASE

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**Background.** Homocysteine [HSH] and cysteine [CSH] are part of the essential amino acid methionine's transsulfuration metabolic pathway; their only physical difference is an extra methylene group in HSH's side chain. Both have a free sulfhydryl group and are mercaptan reagents. They can cleave disulfide bonds in other proteins via their thiolate ions. However, CSH's thiolate ion has a lower pKa value than that of HSH. An important observation is that the so formed new disulfide bond is much more labile for CSH relative to HSH. Therefore other researchers have dismissed the earlier theory of cysteine as source of reactive oxygen species in the pathogenesis of atherosclerosis. In contrast, HSH forms what may be considered an irreversible disulfide bond. For mercaptan reagents to be reactive, the target protein's conformation or tertiary structure must be destroyed. In the arteries the target proteins for HSH reactions may be large proteins such as extracellular matrix proteins (proteoglycans, fibronectin) and cell-to-cell adhesive molecules (integrins and cadherins) of the endothelium. These proteins have large loops with only a few disulfide bonds and are usually intact under normal blood flow. However, under haemodynamic stress, their tertiary structure may be affected in such a way that they become suited for deleterious disulfide exchange, even at normal plasma homocysteine levels, to cause increased permeability. At very high plasma levels of homocysteine, we propose that HSH will lead to

desquamated endothelium with the formation of fibrous plaques. At normal or moderately increased levels, it will lead to increased permeability, e.g. LDL to cause *fatty streaks*. In genetic homocystinuria and experimental animals *fatty streaks* may also occur, but further away from large arteries where haemodynamical conditions are less stressed.

**Conclusions.** this theory demonstrates how HSH acts as major causative factor at normal as well as elevated levels in CVD.

### P033

#### HOMOCYSTEINE AS A MAJOR CAUSATIVE FACTOR FOR CARDIOVASCULAR DISEASE AND ALZHEIMER'S DISEASE PART 2: A MECHANISTICAL PROPOSITION FOR ALZHEIMER'S DISEASE

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**Background.** Alzheimer's disease (AD) is known to be related to Amyloid precursor protein and its smaller peptide A-Beta, which consists of 40-42 amino acids. A-Beta is the major component of the neuritic amyloid plaque. It is also known that there are similarities between Alzheimer's disease and Vascular dementia. Many researchers now believe there is a common underlying neuropathology. Some have even suggested that Alzheimer's Disease is a vascular disorder. De la Torre has posited the following causal pathway: Advanced aging and vascular risk factor, Brain hypoperfusion, Neurological energy crisis, Mild cognitive impairment, Neuro degeneration, AD. In addition to its association with cardiovascular disease (CVD), it has also become known that homocysteine is likely to be involved in AD. This raises the interesting question: is homocysteine the common vascular risk factor that damages blood vessels and neurons, or is it a marker for deficiency or depletion of other interconnected molecules or both? We propose that homocysteine has a similar pathological role in brain arteries as in CVD (see previous abstract). In the presence of stress factor(s), it will cause structural protein damage and increased permeability of the endothelium. This damage may initiate events that lead to arterial hypoperfusion. However, because of the physical and molecular structure of brain arteries, the ensuing permeability due to homocysteine's deleterious disulfide exchange reactions may also cause penetration of small as well as large proteins. Some may have proteolytic activity and be involved in the formation of the amyloid plaque. Theoretically, there is also the possibility that homocysteine may directly be involved in cleaving some of the disulfide bonds of the amyloid protein.

**Conclusions.** Our theory proposes how homocysteine is involved in brain hypoperfusion as well as the formation of plaques involved in AD.

### P034

#### HYPERHOMOCYSTEINEMIA ACCELERATES CAROTID ARTERY THROMBOSIS IN MICE

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Hyperhomocysteinemia is a risk factor for thrombosis and stroke, but the mechanisms are not well defined. We tested the hypothesis that hyperhomocysteinemia accelerates

carotid artery thrombosis induced by photochemical injury in mice. Mice heterozygous for a targeted disruption of the cystathionine R-synthase (Cbs) gene (Cbs<sup>+/-</sup>) and wild type littermates (Cbs<sup>+/+</sup>) were fed either a control diet or a high methionine/low folate (HM/LF) diet for 8 months. Plasma total homocysteine (tHcy) was elevated by the HM/LF diet compared with the control diet in both Cbs<sup>+/+</sup> mice (14.8±1.1 vs. 3.7±0.24 μmol/L; *p*<0.05) and Cbs<sup>+/-</sup> mice (50.4±5.4 vs. 7.1±0.6 μmol/L; *p*<0.001). The time to stable occlusion after photochemical injury (rose bengal and green laser) of the carotid artery was approximately 50% shorter in mice fed the HM/LF diet (17.5±2.3 and 18.2±4.0 minutes for Cbs<sup>+/+</sup> and Cbs<sup>+/-</sup> mice, respectively) compared with mice fed the control diet (44.0±9.1 and 31.4±4.7 minutes for Cbs<sup>+/+</sup> and Cbs<sup>+/-</sup> mice, respectively; *p*<0.001). Platelet activation and procoagulant activity in response to thrombin and convulxin did not differ between any groups of mice. Carotid artery occlusion times were not shortened in endothelial nitric oxide synthase null (Nos3<sup>-/-</sup>) mice, which suggested that decreased bioavailability of endothelium-derived NO was not responsible for accelerated thrombosis seen in hyperhomocysteinemic mice. Using real time PCR, we observed a 40% decrease in mRNA levels of the endothelial antithrombotic molecules, thrombomodulin (TM) and endothelial protein C receptor (EPCR) in the lungs of mice fed the HM/LF diet compared with mice fed the control diet (*p*<0.01). Plasma tHcy correlated negatively with mRNA levels for both TM and EPCR (R=0.51, *p*<0.01, and R=0.36, *p*<0.05 respectively). Using the oxidative dye, dihydroethidium (DHE), we detected increased production of superoxide in the carotid arteries of Cbs<sup>+/+</sup> or Cbs<sup>+/-</sup> mice fed the HF/HM diet compared with mice fed the control diet. We conclude that hyperhomocysteinemia increases susceptibility to experimental thrombosis in mice by a mechanism that is independent of platelet activation or endothelium-derived NO, but may involve oxidative stress and decreased expression of TM and EPCR.

### P035

#### HOMOCYSTEINE STIMULATES THE ADHESION OF MONOCYTES TO ENDOTHELIAL CELLS AND THEIR DIFFERENTIATION INTO CHOLESTEROL SCAVENGING MACROPHAGES

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**Background and Aims.** Monocyte (MO) invasion into the vessel wall and their differentiation into macrophages is crucial for plaque development. We investigated whether incubation of cultured MO with Hcy promotes these events, and whether aged garlic extract (AGE), a phytotherapeutic with antioxidant capacities, prevents foam cell differentiation.

**Methods.** THP-1 (human monocytic leukemia) cells were incubated with D,L-Hcy and other substances. Their adhesion to cultured human endothelial cells (EC, EA.hy 926 cells) was studied under static conditions. Expression of beta2-integrins Mac-1alpha and LFA-1beta (ligands for adhesion molecules on EC), and of CD36 (class B scavenger receptor) in the presence of PMA was measured by FACS.

**Results.** Incubation of MO with 200 microM Hcy for 6 hours leads to a significant 2,5 to 3fold increase in adhesion to EC. This is mediated by upregulation of Mac-1alpha and LFA-1beta on MO, as Hcy-incubation significantly increases their surface expression (35 to 45%), and as Hcy-induced MO-adhesion can be blocked with antibodies against beta2-integrins. Hcy-induced MO adhesion to EC may involve

increased cellular oxidant stress as TIRON (superoxide scavenger) partly blocks Hcy's effects. Incubation of PMA stimulated MO with Hcy (10 to 200 microM for 72 h) significantly and dose-dependently increases CD36 expression up to 38±5%. This may be mediated by activation of the transcription factor PPARgamma, as a synthetic inhibitor blocks Hcy's effects. Coincubation with AGE (5 mg/mL) significantly suppressed CD36 expression by 62±14% compared to control and by 49±9% compared to Hcy-incubated cells.

**Conclusions.** Hcy enhances MO adhesion to EC via oxidant stress sensitive upregulation of Mac-1alpha, LFA-1beta and enhances PMA-induced CD36 expression and foam cell formation via the PPARgamma pathway. This suggests that Hcy could promote the formation and development of early atherosclerotic lesions, while AGE could be useful in its prevention.

### P036

#### ACUTE HYPERHOMOCYSTEINEMIA INDUCES MACRO- AND MICROVASCULAR ENDOTHELIAL DYSFUNCTION THAT CAN BE REVERSED BY TREATMENT WITH AGED GARLIC EXTRACT

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**Background.** We studied the effects of acute hyperhomocysteinemia on macro- and microvascular endothelial function in healthy volunteers, and the effect of aged garlic extract (AGE), a phytotherapeutic with antioxidant capacities, in a placebo-controlled double-blinded cross-over study.

**Methods and Results.** Oral methionine challenge (0.1 g per kg body weight) increases plasma homocysteine levels within four hours (from 6.1±3.7 to 31.5±8.4 micromol/L). Acute hyperhomocysteinemia (HHcy) disturbs endothelial function in conductance vessels as indicated by a significant decrease in flow-mediated, endothelium-dependent vasodilation of the brachial artery as measured by vascular ultrasound (from 8.0±4.7% vasodilation before to 3.1±2.4% vasodilation during HHcy). Endothelium-independent, nitroglycerin-induced vasodilation is not affected by HHcy. Microvascular endothelial dysfunction was assessed by measuring forearm skin perfusion before and after iontophoresis of acetylcholine using laser doppler fluxmetry. Acetylcholine results in a 9.8±3.2 fold increase in skin perfusion before HHcy, which is significantly reduced to a 7.5±2.4 fold increase during HHcy. Microvascular endothelial dysfunction is further indicated by a significant decrease in the number of finger tip capillaries that are recruited during postischemic hyperemia and visualized by capillary microscopy (from 31.5±19.9 before to 19.0±10.2 recruited capillaries per microscopic field during HHcy, respectively).

Pretreatment of AGE for six weeks results in a significant increase in flow-mediated vasodilation of the brachial artery during HHcy from 3.6±2.3% during Placebo to 6.0±2.2% during AGE treatment. After AGE treatment, HHcy no longer induces microvascular endothelial dysfunction (increase in acetylcholine-induced skin perfusion before HHcy 9.1±4.2 fold compared to 9.0±4.7 fold during HHcy).

**Conclusions.** Acute hyperhomocysteinemia induces endothelial dysfunction in conductance as well as in resistance vessels and AGE at least partly compensates for the adverse effects of homocysteine on endothelial function.

P037

**HOMOCYSTEINE AND INFLAMMATION IN HEART FAILURE**

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Heart failure (HF) has been considered for several years as a hemodynamic disorder or as the result of different neuro-humoral disorders. Recently, experimental data have demonstrated the presence of high levels of proinflammatory cytokines, suggesting an "inflammatory hypothesis" in the pathophysiology of HF. In addition, data are available on the possible role of homocysteine (Hcy) levels in the ventricular remodeling of patients with heart failure. Aims of our study were: 1) to investigate the role of Hcy and inflammatory markers in patients with HF; 2) to evaluate their relationship with markers of ventricular remodeling such as metalloproteinases (MMP). We studied 79 patients with a diagnosis of HF (63 M/ 16 F; 74 (43-95 yrs) and 79 healthy subjects as controls, comparable for age and sex (63 M/ 16 F; 74 (40-83) yrs). 27 patients were in class NYHA II, 30 were in class NYHA III and 22 in IV. We determined fasting Hcy levels by FPIA method; interleukin-6 (IL6) by an ELISA method; C reactive protein (CRP) levels by a nephelometric high sensitivity assay; and MMP3 and 9 (total activity) by immunoenzymatic assay. Hcy levels were significantly higher in patients with respect to controls [14,2 (6.5-41.5) micromol/L versus 8.7 (5.1-24) micromol/L;  $p < 0.001$ ]. Thirty-four out of seventy-nine patients (43%) had Hcy above 15 micromol/L. Hcy levels were significantly higher in class NYHA IV [16.7 (9.9-33.4) micromol/L] with respect to class III [13.9 (6.5-41.5) micromol/L;  $p < 0.05$ ] and class II [12.5 (7.9-32.6) micromol/L;  $p < 0.01$ ]. Hcy levels were significantly different in relation to the etiology of HF (hypertensive: 17.9 (7.4-41.5) micromol/L; dilatative: 15.3 (6.5-27.8) micromol/L; ischemic: 12.7 (6.7-33.4) micromol/L. In addition, Hcy levels were significantly higher in patients with severe systolic dysfunction of left ventricular measured by the ejection fraction (EF): EF  $> 40\%$  = 12.5 (6.7-30) micromol/L; EF 30-40% = 13.6 (6.5-41.5) micromol/L; EF  $< 30\%$  = 15 (8.2-33.4) micromol/L;  $p$  for trend  $< 0.05$ . Finally, Hcy was significantly higher in patients with diastolic dysfunction (diagnosed on an altered ecocardiographic E/A ratio): 15.5 (6.5-41.5) micromol/L versus 12.6 (6.7-27.8) micromol/L;  $p < 0.01$ .

CRP and IL6 levels were significantly higher in patients than in controls [CRP: 10 (0.7-21) mg/L versus 1.9 (0.8-5.4) mg/L,  $p < 0.01$ ; IL6: 8.9 (0.9-51.5) pg/mL versus 4.6 (0.5-12) pg/mL,  $p < 0.01$ ]. We demonstrated a significant correlation between Hcy and IL6 ( $r = 0.31$ ;  $p < 0.01$ ), Hcy and CRP ( $r = 0.54$ ;  $p < 0.001$ ). We documented a significant correlation between Hcy and MMP-3 and -9 only in the subgroup ( $n = 34$ ) of patients with hyperhomocysteinemia (i.e. Hcy levels above 15 micromol/L) ( $r = 0.46$ ;  $p = 0.009$ ). In addition, we found a significant correlation between inflammation and MMP (CRP-MMP9:  $r = 0.31$ ;  $p < 0.01$ ; CRP-MMP3:  $r = 0.44$ ,  $p < 0.005$ ; IL6-MMP9:  $r = 0.35$ ,  $p < 0.05$ ; IL6-MMP3:  $r = 0.33$ ,  $p < 0.005$ ).

These results demonstrate a high prevalence of hyperhomocysteinemia in HF, which is associated with the impairment of systolic and diastolic ventricular function and with the etiology of HF. The evidence of a proinflammatory state in HF which is associated with Hcy levels and the first *in vivo* demonstration of a correlation between hyperhomocysteinemia and MMP, which are involved in the ventricular remodeling, prompt clinical trials addressed to evaluate the use of vitamin supplementation and anti-inflammatory therapy in HF patients.

P038

**HYPERHOMOCYSTEINEMIA IS A RISK FACTOR FOR IDIOPATHIC SUDDEN SENSORINEURAL HEARING LOSS**

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Over the last years there has been a significant increase of sudden hearing loss (SHL) in Western countries with an incidence of 20 out of 100,000 persons affected every year. However, no clear pathogenetic causes for this disease have been found thus far in patients in whom an infectious episode or acoustic neurinoma have been excluded.

Aim of this study was to investigate a number of acquired and inherited thrombophilic risk factors (lipoprotein(a); fasting homocysteine; plasminogen activator inhibitor-1; antiphospholipid antibodies; antithrombin, protein C and S; factor V Leiden, factor II polymorphism) in patients (pts) with idiopathic SHL. We investigated 168 consecutive pts (M/F; age: 55 (19-79) yrs) with a diagnosis of idiopathic SHL within 30 days from the onset of symptoms, and 168 controls (ctrl) (M/F; age 54 (19-78) yrs). In no pt a deficiency of physiological clotting inhibitors antithrombin, protein C and protein S was found. No significant differences between pts and ctrl were observed for Lp(a) plasma levels [116 (1-817) mg/L vs 102 (9-695) mg/L] and for the presence of FV Leiden (4.2% vs 4%) and factor II polymorphisms (3% vs 2.4%). In pts fasting homocysteine levels were significantly ( $p$  at least  $< 0.05$ ) higher than in controls (12 (6.8-60) micromol/L vs 8.7 (5.1-24) micromol/L) as well as PAI-1 levels [19 (2-43.7) IU/mL vs 8 (4.1-17.4) IU/mL]. The positivity for antiphospholipid antibodies (lupus anticoagulant and/or anticardiolipin antibodies) was significantly more frequent in pts than in ctrl (31% vs 3%;  $p < 0.001$ ). At multivariate analysis independent risk factors for ISSHL were: elevated PAI-1 levels (OR 13.2 (95% CI 5.1-34.3);  $p < 0.000$ ); antiphospholipid antibodies (OR 9.3 (95% CI 2.9-30.1);  $p < 0.000$ ); hyperhomocysteinemia (OR 8.4 (95% CI 3.2-22.2);  $p < 0.000$ ).

These preliminary data suggest that thrombophilia could play a role in idiopathic SHL, supporting the hypothesis of a vascular occlusion in its pathogenesis.

P039

**HYPERHOMOCYSTEINEMIA AS AN INDEPENDENT MARKER OF OCCULT CORONARY AND PERIPHERAL ARTERIAL DISEASES IN PATIENTS WITH SYMPTOMATIC INTRACRANIAL ATHEROSCLEROSIS**

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**Background.** Patients affected by symptomatic intracranial atherosclerosis are exposed to a high risk of suffering atherothrombotic ischemic events in the coronary and peripheral circulations. **Aims.** We tested the hypothesis whether homocysteine (Hcy) may serve as a marker of not overt coronary artery disease (CAD) and peripheral arterial disease (PAD) in this group of stroke patients. **Methods:** From 186 first-ever transient ischemic attack (TIA) or ischemic stroke patients with intracranial atherosclerotic stenoses, 65 fulfilled selection criteria, including absence of known CAD. All patients underwent a maximal-stress myocardial perfu-

sion SPECT. An ankle-brachial index (ABI) <0.9 was considered indicative of PAD. Plasmatic Hcy was determined previous to SPECT performance and ABI measurement.

**Results.** Forty-three patients presented with an ischemic stroke and 22 with a TIA. Magnetic Resonance Angiography confirmed 175 intracranial stenoses. Stress-rest SPECT detected reversible myocardial perfusion defects in 34 (52%) patients. An ABI <0.9 was present in 42 (69%) patients. Hcy level was higher in patients with a pathologic myocardial SPECT ( $p=0.038$ ), and a negative correlation was found between Hcy and ABI values ( $r=-0.363$ ,  $p=0.008$ ). Logistic regression models identified a high Hcy level as an independent marker of both silent myocardial ischemia (OR 3.9, 95% IC 1.1-14.1,  $p=0.03$ ) and PAD (OR 13.3, 1.4-120.9,  $p=0.02$ ), after adjustment by age, sex and traditional risk factors.

**Conclusions.** Hyperhomocysteinemia is an independent marker of occult CAD and PAD in patients with symptomatic intracranial large-artery atherosclerosis.

#### P040

##### HOMOCYSTEINE IS A RISK FACTOR FOR STROKE IN FINNISH WOMEN: A 15-YEAR PROSPECTIVE ANALYSIS

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Elevated plasma homocysteine (Hcy) is considered a risk factor for coronary heart disease, but evidence regarding the risk of stroke is equivocal. The aim was to find out if there is an association between serum Hcy and stroke in a prospective analysis. The subjects were nested in a random population sample of 4363 men and 4548 women aged 30-59 years at baseline, who participated in a cardiovascular (CVD) risk factor survey in eastern Finland in 1977. Frozen serum samples were available for 58 men and 51 women with history of CVD, and 490 men and 498 women free of CVD at baseline, who were followed for 15 years.

At baseline the mean (SD) serum Hcy concentrations in women with and without history of CVD were 12.3 (2.9) mmol/L and 10.8 (3.4) mmol/L ( $p=0.001$ ), respectively. The multivariate adjusted odds ratio (OR) for stroke for the highest tertile vs. the lowest was 3.41 (95% CI, 1.33-8.74). In the CVD-free women at baseline, the mean Hcy concentration was lower in those remaining CVD-free ( $n=263$ ) than in those with an incident stroke event ( $n=193$ ) during the follow-up, 10.4 (3.0) mmol/L vs. 11.4 (4.1) mmol/L ( $p=0.004$ ). The multivariate OR for incident stroke was 2.33 (1.44-3.77,  $p<0.001$ ) comparing the highest quartile with the lower ones. In men, neither baseline nor follow-up concentrations differed between cases and controls. In conclusion, serum Hcy concentration predicted incident stroke in women but not men.

#### P041

WITHDRAWN

#### P042

##### STAGE II OF THE CARDIOVIT STUDY: CARDIOVIT, ATHEROSCLEROTIC VASCULAR DISEASE AND HYPERHOMOCYSTEINEMIA: AN EPIDEMIOLOGICAL STUDY IN INDIANS, ADDITIONALLY EVALUATING THE EFFECT OF ORAL VITAMIN SUPPLEMENTATION

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**Background.** Hyperhomocyst(e)inemia (HypertHcy) is emerging as a novel independent risk factor for atherosclerotic vascular disease (AVD) in Indians possibly because of inadequate folic acid and vitamin B12 status. The exact dose of folic acid to reduce plasma homocysteine (tHcy) is a matter of debate.

**Aims.** The primary objective was to evaluate the tHcy lowering effect and safety of Cardiovit: a formulation containing folic acid (300  $\mu$ g), vitamin B12 (1  $\mu$ g), vitamin B6 (1.5 mg), vitamin A (2500 IU), vitamin C (50 mg), vitamin E (10 IU) and selenium (100  $\mu$ g). The secondary objective was to determine if hypertHcy is linked to folic acid and vitamin B12 deficiencies.

**Methods.** Randomized 6-week prospective three arm, double-blind, double-dummy, placebo-controlled, parallel-group comparative study. Subjects with tHcy > 12 mmol/L and not on any other multivitamin supplements or drugs interfering with folic acid metabolism and with normal liver and renal function were included. They were divided into three groups (Group 1: two tablets of placebo;  $n=62$ , Group 2: one tablet of Cardiovit and placebo each;  $n=65$ , and Group 3: two tablets of Cardiovit;  $n=57$ ) tHcy, serum folic acid, red cell folate and serum vitamin B12 was done at baseline and repeated at 6 weeks.

**Results.** Baseline values of tHcy were similar in three groups. At week 6, there was a significant reduction in tHcy compared to baseline in all the groups. The reduction in tHcy was significantly greater in Group 2 ( $-7.59 \pm 7.97$   $\mu$ mol/L) and Group 3 ( $-7.00 \pm 6.03$   $\mu$ mol/L) compared to Group 1 ( $-3.36 \pm 5.79$   $\mu$ mol/L). There was no significant difference in fall in tHcy between Group 2 and 3. A rise in serum folic acid and red cell folate was seen in Group 2 ( $6.48 \pm 3.9$  ng/mL and  $65.91 \pm 78.85$  ng/mL) and Group 3 ( $9.57 \pm 6.56$  ng/mL and  $89.42 \pm 115.46$  ng/mL) but not in Group 1. There was no difference in serum vitamin B12 between the three groups. Treatment-emergent adverse events were similar in all the three groups. tHcy at baseline showed a negative correlation with baseline levels of serum vitamin B12, serum folate and RBC folate. Change in tHcy levels over 6 weeks showed a negative correlation with change in levels of serum folate and RBC folate.

**Conclusions.** Cardiovit once a day (folic acid=300  $\mu$ g) was as effective as twice a day (folic acid=600  $\mu$ g) in reducing tHcy and increasing serum and red cell folate over a 6-week period.

Trademark of Emil Pharmaceuticals Industries Pvt Ltd, Marketed by Pfizer Ltd, India

## P043

**HIGH LEVELS OF HOMOCYSTEINE ARE ASSOCIATED WITH SMALL NUMBER OF TRADITIONAL CARDIOVASCULAR RISK FACTORS IN PATIENTS WITH A SIMILAR DEGREE OF CORONARY ATHEROSCLEROSIS**

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**Background.** Since 1969, with McCully's studies, the association between very high levels of homocysteine (>100 micromol/L) and an elevated risk of premature cardiovascular disease is well established. Whether the much more frequent finding of moderate-intermediate increase in plasma total homocysteine (tHcy) causes cardiovascular disease has been subject of debate.

**Aims.** To evaluate the number of coexisting major traditional risk factors within groups with different levels of tHcy but the same degree of coronary artery disease (CAD).

**Methods.** We studied 180 patients with angiographically documented CAD, divided in three groups according to tHcy levels: 60 patients with normal tHcy (<15 mmol/L), 60 patients with moderate and 60 patients with intermediate hyper-homocysteinemia (between 15 and 30 and between 30 and 100 micromol/L respectively). Within the Verona Heart Project, the three groups of patients were sorted in order to be exactly matched for gender, age, degree of CAD ( $p>0.95$ ). In all patients we considered the presence of traditional risk factors for CAD (i.e. hypertension, diabetes, hyperlipidemia, smoking habit, familial history of ischaemic heart disease) and evaluated the determinants of tHcy levels.

**Results.** The population as a whole was subdivided for having a great or small number of traditional risk factors (i.e.  $\leq 3$  and  $>3$ , respectively). The group with  $>3$  risk factors was significantly less represented among patients with high tHcy levels (36.2%, 21.7%, 15.1%, in patients with normal, moderate, intermediate tHcy respectively,  $p=0.029$ ). Folate and vitamin B12 concentrations, creatinine, MTHFR 677C>T polymorphism were the major determinants of tHcy in our population. Noteworthy, creatininemia gradually increased with increasing tHcy levels (90.99, 101.59, 109.87 micromol/L in patients with normal, moderate, intermediate tHcy respectively,  $p=0.001$ ).

**Conclusions.** Among patients with the same degree of coronary artery disease, those with high tHcy concentrations show a lower number of traditional risk factors.

## P044

**FOLATE, VITAMIN B12, AND RISK OF ISCHEMIC AND HEMORRHAGIC STROKE: A PROSPECTIVE, NESTED CASE-REFERENT STUDY OF PLASMA CONCENTRATIONS AND DIETARY INTAKE**

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**Background.** Folate metabolism has been implicated in stroke. However, the possibility of a role for folate and vitamin B12, independent of their effects on homocysteine sta-

tus, remains to be explored.

**Aims.** The aim of this prospective, population-based study was to relate plasma and dietary intake levels of folate and vitamin B12 to risk of stroke, taking into consideration plasma homocysteine concentrations and methylenetetrahydrofolate reductase polymorphisms.

**Methods.** Subjects were 334 ischemic and 62 hemorrhagic stroke cases and matched, double referents from the Northern Sweden Health and Disease Cohort.

**Results.** Plasma folate was statistically significantly associated with risk of hemorrhagic stroke in an inverse, linear manner, both in univariate analysis and after adjustment for conventional risk factors including hypertension (odds ratio (OR) for highest vs. lowest quartile 0.21 (95% CI: 0.06 to 0.71;  $p$  for trend=0.008). Risk estimates were attenuated by inclusion of homocysteine in the model (OR=0.34; 95% CI: 0.08-1.40;  $p$  for trend=0.088). A similar pattern was observed for increasing folate intake (multivariate OR=0.07; 95% CI: 0.01-0.55;  $p$  for trend=0.031 without homocysteine, and OR=0.16, 95% CI: 0.02-1.23;  $p$  for trend=0.118 with homocysteine in the analysis). We found little evidence of an association between plasma or dietary folate and risk of ischemic stroke. Neither plasma nor dietary vitamin B12 was associated with risk of either stroke subtype.

**Conclusions.** The results of this study suggest a protective role for folate, possibly in addition to its effects on homocysteine status, in hemorrhagic but not ischemic stroke.

## P045

**PROSPECTIVE STUDY OF FIRST-EVER STROKE IN RELATION TO PLASMA HOMOCYSTEINE AND MTHFR 677C>T AND 1298A>C GENOTYPES - EVIDENCE OF AN ASSOCIATION WITH HEMORRHAGIC STROKE**

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Abnormalities in homocysteine metabolism have been suggested as risk factors for stroke. The aim of the present study was to examine whether total homocysteine (tHcy) concentration and its main genetic determinant, methylenetetrahydrofolate reductase (MTHFR), were associated with first-ever ischemic or hemorrhagic stroke. We studied prospectively all subjects who had previously donated blood in population-based health surveys in northern Sweden. Stroke cases were defined according to WHO MONICA criteria. Over a median follow-up period of 4 years, 387 subjects developed a first-ever ischemic and 71 subjects a first-ever hemorrhagic stroke. Risk of hemorrhagic stroke increased exponentially through homocysteine quartiles; the odds ratio for highest versus lowest quartile was 12.64 (95% CI 3.00-53.20),  $p$  for trend <0.001. This association remained statistically significant after adjustment for hypertension and BMI. Odds ratios for hemorrhagic stroke for the MTHFR 677 CT and TT genotypes were 2.23 (1.10-4.55) and 2.78 (0.94-8.25), respectively ( $p$  for trend = 0.022). In contrast, the MTHFR 1298A>C polymorphism appeared to be protective. After adjustment for tHcy, BMI, and hypertension, the associations for both MTHFR polymorphisms remained statistically significant. Haplotype analyses confirmed that the MTHFR 677 T<1298 haplotype was posi-

tively related to risk of hemorrhagic stroke. Neither tHcy nor the MTHFR polymorphisms were significant predictors of ischemic stroke. We conclude that both elevated plasma homocysteine and the MTHFR 677C>T polymorphism are indicators of increased risk of hemorrhagic, but not ischemic stroke.

#### P046

### LOW DOSE FOLIC ACID (0.2 MG/D) GIVEN CHRONICALLY IS AS EFFECTIVE AS HIGHER DOSES IN LOWERING HOMOCYSTEINE IN BOTH ISCHAEMIC HEART DISEASE PATIENTS AND HEALTHY CONTROLS

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**Background.** Controversy currently exists regarding the dose of folic acid needed to lower total plasma homocysteine concentrations (tHcy) in ischaemic heart disease patients (IHD).

**Aims.** To determine the minimum effective dose of folic acid required to maximally lower tHcy in IHD patients and healthy age-sex matched controls.

**Methods.** IHD patients (n 101) and controls (n 81) were each stratified on the basis of their screening tHcy and randomised to receive either placebo, 0.2, 0.4 or 0.8 mg folic acid daily for 26 weeks. Fasting blood samples (20 ml) collected at baseline and post-intervention were analysed for tHcy (ImX) and serum folate (microbiological assay).

**Results.** In both IHD patients and controls, an incremental response in serum folate was observed with each increasing dose of folic acid. In contrast, the tHcy response showed a plateau at 0.2 mg/d. After controlling for baseline tHcy, there was no difference in tHcy response between the two groups, therefore the data were combined (see Table 1).

**Conclusions.** A dose of folic acid as low as 0.2 mg/d, potentially achievable by food fortification, can maximally lower tHcy in both IHD patients and healthy controls. Previous studies may have underestimated the effect of a given dose, either because of too short an intervention period or poor subject compliance.

*This research was funded by the Northern Ireland Chest, Heart and Stroke Association.*

**Table 1.**

	Placebo (n=40)	0.2 mg (n=45)	0.4 mg (n=46)	0.8 mg (n=51)
<b>tHcy (&lt;mol/L)</b>				
Week 0	11.1 (9.6, 13.3)	11.6 (9.8, 15.3)	12.1 (9.4, 15.5)	11.7 (9.7, 15.1)
Week 26	11.0 (10.0, 14.6)	10.0 (8.9, 12.0)	10.1(8.5, 12.4)	10.0 (8.4, 12.1)
Response	-0.1 (-0.9, 1.4) <sup>a</sup>	-1.9 (-3.6, -0.7) <sup>b</sup>	-1.6 (-3.1, -0.4) <sup>b</sup>	-1.6 (-4.1, -0.7) <sup>b</sup>
% response	-1.1 (-8.5, 14.7) <sup>a</sup>	-16.5 (-25.7, -6.3) <sup>b</sup>	-14.0 (-22.4, -4.6) <sup>b</sup>	-15.5 (-27.2, -6.8) <sup>b</sup>

Median (IQR). <sup>a,b</sup>Unlike superscripts are significantly different (ANOVA)(P<0.05; Bonferroni).

#### P047

### MILD FOLATE DEFICIENCY INDUCES A PRO-ATHEROSCLEROTIC PHENOTYPE IN CULTURED ENDOTHELIAL CELLS

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A low folate, high homocysteine phenotype is associated with a wide range of human pathologies including atherothrombotic diseases and neural tube defects. However, it remains unclear whether it is low folate, high homocysteine, or a combination of each that is the etiologically important variable. Furthermore, the related pathogenic mechanisms are poorly defined. To study the isolated effect of low folate conditions in a biologically relevant model, we adapted the human endothelial cell line Ea.hy 926 to growth in high folate (HI cells) and low folate (LO cells) conditions. Relative to HI cells, LO cells had a morphological phenotype characterized by more elongated shape, disordered monolayer formation/structure and a profusion of actin stress fibers. The barrier function of LO cells was significantly compromised as evidenced by increased permeability. In addition, LO cells synthesized higher quantities of pro-inflammatory mediators. One of these could be used to induce a *LO cell phenotype* in HI cells in the absence of any manipulation of folate or homocysteine concentrations. Conversely, inhibitors of the signal transduction pathway engaged by this mediator, as well as statin drugs, could reverse the phenotype elicited by low folate growth conditions. Based on the above observations we postulate that suboptimal folate is itself pathogenic, and that it exerts its negative clinical effects by inducing (i) physical changes in the integrity of cell layers that affect gross architecture, permeability, the composition and accessibility of the extracellular matrix, proliferation and motility; and (ii) the synthesis of molecules that actively promote inflammation and chemotaxis. We further postulate that such changes, individually or in combination, are responsible for many of the clinical conditions that are underpinned by folate and homocysteine dysregulation, in particular vascular diseases and neural tube defects.

#### P048

### GENETIC POLYMORPHISMS IN TWO GENES OF THE FOLATE METABOLIC NETWORK, HOMOCYSTEINE AND CARDIOVASCULAR DISEASE RISK

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Variants in genes of the folate metabolic network play an important role in a variety of diseases. Methylene tetrahydrofolate dehydrogenase (MTHFD) is a trifunctional enzyme that catalyzes the sequential interconversion of tetrahydrofolate derivatives required for purine, thymidylate and methionine biosynthesis. A SNP in the MTHFD gene (MTHFD G1958A), specifically the AA genotype, is associated with an increased risk of neural-tube defects and abruptio placentae. We investigated the association between MTHFD and CVD, considering interactions with MTHFR C677T. We assessed the genotype-homocysteine relation and whether the genotype-CVD association was mediated by homocysteine. In a nested case-control study within the

## Homocysteine Metabolism

Normative Aging Study, 507 cases were matched (2:1) to controls. The prevalence of MTHFD GA and AA genotypes was 53.5% and 19.7%. The MTHFD and MTHFR C677T genotypes were in linkage disequilibrium ( $p=0.09$ ). In conditional logistic models adjusted for covariates, MTHFD GA/AA (vs. AA) had a small protective effect on CVD risk (OR 0.8 [95% CI 0.6,1.1]). A gene-gene interaction was found (overall  $p=0.24$ , individual coefficients  $p$  values 0.09 and 0.35). The protective effect of MTHFD GA/AA genotype (vs. GG) was stronger in men with MTHFR C677T CC genotype (OR 0.62 [0.4, 0.96]) compared to men with MTHFR C677T CT (OR 1.0 [0.4, 2.2]) or TT genotype (OR 0.9 [0.3, 2.8]). Serum homocysteine was  $10.6\pm 4.5$  in MTHFD GG men compared to  $10.6\pm 3.1$  in MTHFD GA/AA men. Among MTHFR CC men, the protective effect of the MTHFD A allele on CVD was not mediated by homocysteine: men carrying the MTHFD A allele had a higher mean serum homocysteine ( $10.6\pm 3.1$ ) compared to men with the MTHFD GG genotype ( $9.7\pm 3.0$ ). In summary, a genotype associated with increased risk of poor reproductive outcomes has a protective association with CVD risk, which does not appear to be mediated by homocysteine.

### P049

#### HOMOCYSTINURIC PHENOTYPE IN A YOUNG PATIENT WITH MULTIPLE HETEROZYGOUS MUTATIONS OF METHYLENE TETRAHYDROFOLATE REDUCTASE (MTHFR) AND CYSTATHIONINE BETA-SYNTHASE (CBS): THE EFFECT OF VITAMIN REPLACEMENT ON SYMPTOMATIC ATHEROSCLEROTIC LESIONS

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We discuss the case of a young male (31 years old), non smoker, presenting marfanoid habitus, ectopia lentis and severe and diffuse atherosclerosis (right renal artery stenosis, lower limbs arteriopathy and coronary vessel disease). Laboratory findings showed high Hcy (364 mmol/L), low levels of cyanocobalamin and folic acid, MTHFR polymorphism (heterozygous for both the C677T and A1298C variant), and CBS heterozygous mutation G919A. The predominant CBS mutation (T833C) in homocystinuria was absent. The patient has the wild type T833T genotype. MTHFR activity in cultured fibroblast, in the presence and in the absence of flavine adenine nucleotide (FAD), was within normal range; on the other hand, CBS activity, with and without pyridoxal-5-phosphate, was low-normal but not deficient. Father presented heterozygous MTHFR mutations C677T and A1298C, mild hyperhomocystinemia and slight cyanocobalamin decrease; mother and brother presented heterozygous A1298C variant with normal Hcy. All relatives were asymptomatic for vascular disease. The patient received pyridoxine (50 mg) and folic acid (5 mg) daily plus cyanocobalamin (1000 mcg) monthly. Six years later, the patient appears symptom-free and with normal Hcy levels. Coronary angiography and 2-D B-mode ultrasound scans of the leg revealed complete regression of atherosclerotic lesions.

**Conclusions.** the severe clinical phenotype and the very high Hcy levels are typical expression of homocystinuric condition with homozygous CBS mutation. In our case, multiple heterozygous mutations of the enzymes involved in Hcy metabolism overlaps the clinical picture of homocystinuria. Exceeding the mutations observed, the results of

enzyme activities in cultured fibroblasts also suggest the possible impairment of MS function. The most interesting feature derives from the response to therapy. Vitamin replacement exerted persistent lowering effect on Hcy level and induced clinical and radiological regression of atherosclerotic lesions. The complete recovery of Hcy-mediated vascular damage could be the first step to restoring a normal endothelial function.

**Table 1. MTHFR and CBS activities in cultured fibroblasts (all values are the mean of duplicate fibroblast cultures)**

MTHFR activity (nmol CH20/h.mg protein)	-FAD	+FAD	Remarks
Normal range (N=6)	3.9-8.6	4.9-10.0	
Obligate Heterozygote (N=1)	1.5	3.5	
	0.2	0.7	
Homozygotes (N=3)	0.8	1.5	
	0.5	1.6	
L.O. (patient)	4.3	5.5	Normal activities
CBS activity (nmol cystathionine/h. mg protein)	-PLP	+PLP	Remarks
Control range (N=12)	2.3-18.2	3.46-22.3	
Obligate heterozygotes range (N=13)	0.17-2.4	0.39-5.40	
Homozygotes (N=16)	0.19	0.33	
	0.12	0.22	
	0.14	0.16	
	0.00	0.00	
	0.08	1.03	
	0.00	0.46	
	0.00	0.24	
	0.00	0.24	
	0.00	0.00	
	0.00	0.12	
	0.00	0.12	
	0.00	0.00	
	0.04	1.55	
	0.04	0.38	
	0.06	1.08	
	0.03	0.08	
L.O. (patient)	4.6	9.6	Normal activities

FAD: flavine-adenine nucleotide; PLP: pyridoxal-5-phosphate

## P49a

## HYPERMOCYSTEINAEMIA IN THE ACUTE PHASE AFTER ISCHEMIC STROKE

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**Background and Purpose.** The goals of this work were to investigate whether the plasma homocysteine level (tHcy) in the ischemic acute stroke. Determine if the tHcy is an independent risk factor for recurrent stroke.

**Methods.** We performed a transversal study of 36 patients (mean age 62,5±15 years) with ischemic stroke. Fasting tHcy was measured 14 hours after the primary admission.

**Results.** The mean serum homocysteine level in men were 19,5 umol/L and women 24,9 umol/L; they weren't significant ( $p$  0,141). The age wasn't significant ( $r=0,18$   $p$  0,29) with the tHcy. The correlation between the tHcy and the risk factor: Arterial Hypertension 21,4 umol/L without it 21,4 umol/L ( $p$  0,751). Diabetes 24,1 umol/L and without it 20,6 umol/L ( $p$  0,421), Dyslipidemia 21,0 umol/L and without it 21,5 umol/L ( $p$  0,442), atrial fibrillation 18,9 umol/L and without it 21,8 umol/L ( $p$  0,371), with smoke 19,3 umol/L and without it 21,6 umol/L ( $p$  0,957). With before ischemic stroke 26,2 umol/L and without it 18,6 umol/L ( $p$  0,037). The relation with the NHISS score was significant ( $p$  0,003).

**Conclusions.** The plasma homocysteine level are increased in the acute phase after ischemic stroke. There are correlation between the plasma homocysteine level and recurrent stroke. The serum homocysteine is an independent risk factor for stroke.

**Table 1. Risk Factors and homocysteine.**

Risk factor	Serum homocysteine		p value
	Present	Absent	
Arterial Hypertension	21,4	21,4	0,751
Recurrent Stroke	26,2	18,6	0,037
Diabetes Mellitus	24,1	20,6	0,421
Dyslipidemia	21,0	21,5	0,442
Atrial Fibrillation	18,9	21,8	0,371
Smoke	19,3	21,6	0,957

## P050

## DETERMINANTS OF MMA LEVELS IN BRAZILIAN MOTHER AND NEWBORNS

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Elevated methylmalonic acid (MMA) is a good marker for cobalamin deficiency. The aim of this study was evaluate the determinants of MMA levels in mother and newborns. Genotypes for MTR A2756G and MTRR A66G polymorphisms were determined by PCR-FLRP. The levels of cobalamin, RBC folate, serum folate, MMA and creatinine were determined in 369 pregnant women (37-42 weeks of gestational age) and their newborns. Three models of linear regression analysis with stepwise were analysed (see Table below). We conclude that neonatal MTR 2756AA genotype is a determinant for neonatal MMA along with cobalamin levels.

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**Table 1.**

Dependent Variables N	Independent Variables	B	p value	R <sup>2</sup> Partial
<b>MODEL 1</b>				
Maternal MMA* 295	1. Maternal Creatinine*	0.342	0.019	1.87
<b>MODEL 2</b>				
Neonatal MMA* 282	1. Maternal Cobalamin*	-0.226	<0.001	4.42
<b>MODEL 3</b>				
Neonatal MMA* 282*	1. Neonatal Cobalamin*	-0.187	<0.001	6.36
	1. MTRAG+GG (ref.)	0.000	1.000	
	MTRAA	0.046	0.017	1.92

\*Log transformed variable. The regression models were adjusted by maternal age, ethnic group and parity. Model 1: Maternal variables as determinants of maternal MMA. Independent variables - maternal concentrations of Cbl, serum folate, RBC folate and creatinine, maternal MTR genotypes (AG+GG (reference) versus AA) e MTRR genotypes (AA (reference) versus AG+GG). Model 2: Maternal variables as determinants of neonatal MMA. Independent variables are the same as in model 1. Model 3: Neonatal variables as determinants of neonatal MMA. Independent variables are the same as in model 1, but from the newborns.

## P051

## CLINICAL SIGNIFICANCE OF LOW COBALAMIN STATUS IN HEALTHY ELDERLY: LOW COBALAMIN STATUS IS ASSOCIATED WITH HIGHER RESTING HEART RATES

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Subclinical vitamin B12 deficiency is a common finding among the elderly, however, its clinical significance is unclear. In particular, the effect of subclinical deficiency was not investigated with respect to peripheral and autonomic nerve involvement. It was the aim of the study to investigate whether peripheral and/or autonomic neuropathy is present in healthy elderly with subclinical cobalamin deficiency. Healthy elderly with no history of cardiovascular disease and gastrointestinal tract disease were included. Serum cobalamin, folate, holo-TC II, methylmalonic acid, homocysteine, blood count and safety parameters (creatinine, liver enzymes, TSH, glucose) were measured. Subclinical cobalamin deficiency was defined by the holo-TC II level (cut-off: 40 pg/mL). Sensitive neurological tests were performed in a double-blind manner in 16 subjects with holo-TC II levels <40 pmol/L and 16 age- and sex-matched controls with holo-TC II levels /40 pmol/L. Peripheral sensory neuropathy was measured as thermal and vibratory distinction on hand and foot. Cardiovascular autonomic function was assessed by the measurement of heart rate variation. Heart rate, heart rate variation and spectral analysis of the low, mid and high frequency range at rest and mean circular resultant during deep breathing and the lying-to-standing maximum/minimum ratio were determined. Serum cobalamin, holo-TC II, MMA and homocysteine differed significantly between groups. Systolic blood pressure was slightly higher in the the cobalamin-deficient group. Cobalamin-deficient subjects showed a 2°C higher warm sensation threshold than control subjects ( $p=0,033$ ) although the mean warm sensation threshold of the cobalamin-deficient group was still within the age related normal range. Mean resting heart rates were significantly higher in subjects with low serum cobalamin levels. Resting heart rate and serum cobalamin were significantly associated ( $r=-0,43$ ). These results suggest that mild cobalamin deficiency may have significant impairments in the autonomic nerve system.

## P051a

**A NON-RADIOACTIVE VITAMIN B12 ABSORPTION TEST EVALUATED IN PATIENTS WITH INHERITED VITAMIN B12 MALABSORPTION, THEIR PARENTS AND HEALTHY CONTROLS**Bor MV,<sup>1</sup> Çetin M,<sup>2</sup> Aytaç S,<sup>2</sup> dem Altay Ç,<sup>2</sup> Nexø E<sup>1</sup><sup>1</sup>Department of Clinical Biochemistry, NBG, AS, Aarhus University Hospital Denmark; <sup>2</sup>Department of Hematology, Children Hospital of Hacettepe University Ankara, Turkey

**Background and Aim.** We recently described a vitamin B12 absorption test based on measurement of an increase in the circulating level of transcobalamin saturated with cobalamin (holoTC) 24 hours after ingestion of vitamin B12. Hereby, we evaluated this test named CobaSorb in 17 Turkish patients with hereditary juvenile megaloblastic anemia (Imerslund-Grasbeck syndrome (n=13) and hereditary intrinsic factor deficiency (n=4)) (patients), their biological parents considered to be heterozygote (n=19) (parents) and healthy controls (n=44) (controls).

**Methods.** We removed blood samples prior to and 24 hours after oral administration of three times 9-µg vitamin B12 and analysed the samples for cobalamins and holoTC. **Results.** After vitamin B12 load, holo-TC and cobalamins increased (Ø increase as median and (range)) alike in controls (26 (-6+63)), (41(-37-109)) and parents (23 (-2+47)), (27 (-15+94)), but statistically less in patients (1(-42+5)), (-3(-32+22)), pmol/L respectively. We analysed the increase in holoTC for the patient group as compared to the control group employing a receiver operating curve (ROC). The areas under ROC curves were 0.97 [(0.93–1.0 (95%CI)] for holoTC and 0.86 (0.77–0.95) for cobalamin.

**Conclusions.** We show that measurement of holoTC after an oral dose of vitamin B12 is superior to measurement of cobalamins for identifying patients who can not absorb vitamin B12.

## P052

**CIRCADIAN VARIATION OF HOLOTRANSCOBALAMIN, TOTAL TRANSCOBALAMIN, TRANSCOBALAMIN SATURATION AND COBALAMINS IN PLASMA**Hvas AM,<sup>1</sup> Gravholt CH,<sup>2</sup> Nexø E<sup>1</sup><sup>1</sup>Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus; <sup>2</sup>Medical Department M (Endocrinology and Diabetes), Aarhus University Hospital, Aarhus, Denmark

One of the common reasons for elevated plasma total homocysteine (tHcy) is vitamin B12 deficiency. Recently, new methods for measurement of holotranscobalamin (holoTC) and the related transcobalamin saturation (TC saturation) have been introduced for diagnosing vitamin B12 deficiency. The aim was to examine the circadian variation of the vitamin B12 related markers and the association between intake of food and fluctuation of holoTC and total TC. The study population consisted of 17 healthy women (mean age, 33 years; range, 24-40 years). The subjects were admitted at 08:00 h after an overnight fast, and from 12:00 h blood samples were obtained every 20 minutes for 24 hours. The subject received meals served at the hospital at 12:30, 15:00, 18:00, 21:00, and 08:00 h. HoloTC and total TC were measured by an ELISA modified to allow the use of an automated ELISA analyser (BEP-2000; Dade Behring). The TC saturation was calculated; holoTC/total TC. All subjects except one had 12:00 h levels within the reference interval for each analysis, and all subjects had normal renal function as judged from plasma creatinine. We found a considerable inter-individual variation in the level of plasma cobalamins

and the related parameters. During the night, the absolute values of all components showed a decrease, but this decrease disappeared after albumin correction. We found no systematic association between intake of food and fluctuation of plasma cobalamins and the related markers. In conclusion plasma cobalamins, holoTC and TC saturation decreased during night, however, this decrease disappeared after correction for plasma albumin. None of the vitamin B12 related markers were substantially influenced by food intake.

## P052a

**A DAILY INTAKE OF AROUND 6 µG VITAMIN B12 APPEARS TO SATURATE ALL THE VITAMIN B12 RELATED PARAMETERS IN DANISH POSTMENOPAUSAL WOMEN**Bor MV,<sup>1,2</sup> Lydeking-Olsen E,<sup>3</sup> Møller J,<sup>4</sup> Nexø E<sup>1</sup><sup>1</sup>Dept. Clinical Biochemistry, NBG, AS; <sup>2</sup>Dept. Clinical Biochemistry, Aalborg Hospital; <sup>4</sup>Dept. Clinical Biochemistry, SKS, all Aarhus University Hospital, Denmark; <sup>3</sup>Institute for Optimum Nutrition, Copenhagen, Denmark

**Background and aim.** The recommendations for daily intake of vitamin B12 vary between 2-6 µg. Hereby, we examined the associations between vitamin B12 intake and markers of vitamin B12 deficiency.

**Methods.** We studied 98 Danish postmenopausal women (age 41-75y). Serum cobalamin, transcobalamin saturated with vitamin B12 (holoTC), TC saturation (holoTC/total TC), methylmalonic acid (MMA) and homocysteine (tHcy) were used to assess vitamin B12 status. The dietary intakes of vitamin B12 were determined from seven-day weighed food records.

**Results.** The total intake of vitamin B12 ranged from 1.2 µg to 23.9 µg/d. All vitamin B12 related parameters correlated significantly with vitamin B12 intake. Those taking supplements (54%) had higher circulating levels of cobalamin and TC saturation, and lower levels of MMA and tHcy than those not taking supplements. All the subjects were divided into quintiles ranked by their total vitamin B12 intake. For all the parameters analyzed, the curves appeared to level off at daily intake of around 6 µg, showing medians and (25% and 75% percentile), n=58 of cobalamin (pmol/L) 380 (270-480), holoTC (pmol/L) 119 (92-162), MMA (mmol/L) 0.12 (0.14-0.17) tHcy (µmol/L) 9.75 (8.3-11.40).

**Conclusions.** A daily vitamin B12 intake of 6 µg appears to be sufficient to correct all the vitamin B12 related parameters.

## P053

**FOLATE VITAMER DISTRIBUTION IN SEVERE COBALAMIN DEFICIENCY: A CASE FOR THE METHYLFOLATE TRAP HYPOTHESIS**

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**Background.** In patients with cobalamin deficiency, it has been hypothesized that DNA biosynthesis defects occur because folate metabolism comes to a virtual hold due to the inability to convert 5-methyltetrahydrofolate either to tetrahydrofolate or back to methylenetetrahydrofolate (the 'methylfolate trap hypothesis'). This hypothesis has never been confirmed by *in vivo* human data. We recently saw a patient with severe cobalamin deficiency, who also was homozygous for the MTHFR 677 C>T mutation. Patients with the 677 TT genotype are known to have a 'natural tendency' to divert folate metabolism away from 5-methylte-

trahydrofolate formation, at least in the erythroid cell line. *Methods.* At baseline and after 3 months intramuscular supplementation with hydroxycobalamin, folates in whole blood lysates were deconjugated in ascorbic acid solutions and, after deproteinization, purified using folate binding protein affinity columns, and concentrated by SPE and evaporation. After LC separation on a C18 column, folates were detected using positive electrospray ionization MS/MS under multiple reaction monitoring conditions.

*Results.* At baseline, serum cobalamin was 46 pmol/L (normal value >150 pmol/L), increasing to 307 pmol after supplementation. Plasma tHcy decreased from 52.9 to 16.8  $\mu$ mol/L. The following alterations in folate vitamers content were observed in whole-blood samples: total folate increased from 121.1 to 225.5 nmol/L, 5-methyltetrahydrofolate increased from 114.4 to 151.9 nmol/L, and total non-methyltetrahydrofolate (sum of methenyltetrahydrofolate and formylated tetrahydrofolates) increased from 6.6 to 73.6 nmol/L. The fraction of non-methylfolates relative to total folate increased from 5.5% before, to 32.6% after supplementation of cobalamin.

*Conclusions.* In this MTHFR 677 TT patient, cobalamin deficiency resulted in an increased tendency to form 5-methyltetrahydrofolate relative to other, non-methyltetrahydrofolate vitamers. In addition, cellular retention of 5-methyltetrahydrofolate was impaired. These data provide unique *in vivo* proof supporting the *methylfolate trap hypothesis*.

#### P053a

##### POST PARTUM STEADY PLASMA COBALAMIN LEVELS REFLECT OPPOSITE CHANGES IN SERUM HOLOTRANSCOBALAMIN (HOLOTC) AND HAPTOCORRIN (HOLOHC). A NINE-MONTH LONGITUDINAL STUDY OF LACTATING WOMEN

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*Background.* The concentration of cobalamin and its binding proteins changes during pregnancy, but little is known concerning the changes occurring post partum.

*Materials and methods.* Lactating mothers (n=89) including 23 supplemented with vitamin B12 (1-18  $\mu$ g/daily), 41 partly supplemented and 25 not supplemented. Blood samples collected after 3 weeks (baseline), 4 and 9 months post partum were analyzed for cobalamins, and the cobalamin binding proteins; transcobalamin (TC) and haptocorrin (HC). Both the total concentration and the cobalamin-saturated form (holo) of TC and HC were analyzed.

*Results.* No significant difference was observed for serum cobalamin or its binding proteins associated with supplementation with vitamin B12 or related to the duration of lactation. Serum cobalamins remained unchanged from 3 weeks to 9 months post partum. Total TC (holoTC) (median  $\pm$  SE pmol/L) decreased between 3 weeks [(709 $\pm$ 23, (87 $\pm$ 12) and 9 months (601 $\pm$ 21, (76 $\pm$ 11) ( $p$ <0.0001, ( $p$ =0.001) while total HC (holoHC) increased from (411 $\pm$ 12 (307 $\pm$ 9) at 4 months to (455 $\pm$ 13(330 $\pm$ 10) to 9 months post partum ( $p$ <0.0001 ( $p$ =0.008)].

*Conclusions.* We report a decrease in TC and an increase in HC during a 9-month period post partum. No difference was observed between the vitamin B<sub>12</sub> supplemented and the unsupplemented group. Thus, supplementation with vitamin B<sub>12</sub> has no impact on the circulating level of cobalamins or its binding proteins in a Danish population of lactating mothers.

#### P054

##### DIAGNOSTIC SCHEME FOR THE EARLY DIAGNOSIS OF VITAMIN B12 DEFICIENCY IN ELDERLY PEOPLE

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*Background.* The elderly population is particularly at risk for developing vitamin B12-deficiency. Inadequate diets, diseases like gastritis or intestinal malabsorption are the main reasons for vitamin B12-deficiency in this age group. Sensitive methods for early detection of vitamin B12-deficiency are not common in the routine laboratory. Normal serum cobalamin (SCbl) does not necessarily reflect a normal B12 status. The determination of methyl malonic acid (MMA) is very expensive and not routinely possible. Holotranscobalamin II (Holo-TC II), the fraction of functional vitamin B12, has been proposed as a more sensitive B12 status indicator. The aim of the present study is to establish a diagnostic scheme (DiagS) taking into account both clinical and economical criteria.

*Material and Methods.* Two-hundred and eighteen elderly of both sexes, aged 60-105, living in an Elderly Home in Granada (Spain), were screened for SCbl (Abbott, IMx), (Holo-TC II) (HoloTC RIA, Axis-Shield, MMA (MS-GC), serum folate, red blood cell (RBC) folate, and tHcy (Abbott, IMx), which were the variables chosen based on clinical criteria.

*Results.* Two alternative decision trees were formulated organizing the before-mentioned parameters in different order. DiagS 1 started with SCbl and included 14 possible outcomes for diagnosing B12 deficiency. DiagS 2 started with Holo-TC II and included 12 possible outcomes for diagnosing B12 deficiency. DiagS 1 identified 8.7% pure B12 deficiency, 50.9% combined B12/folate deficiency, 31.2% pure folate deficiency and 6.0% as being normal. In DiagS 2 the respective values were 9.6%, 67.0%, 14.7% and 5.0%.

*Conclusions.* The proposed diagnostic scheme which eliminates SCbl and starts the screening with Holo-TC II seems to be more precise in detecting functional vitamin B12 deficiency and the combined folate/B12 deficiency in institutionalized elderly. The reason for the different number in each of the deficiency groups in both DiagS has to be analysed more in depth.

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#### P054a

##### RECOMBINANT HUMAN INTRINSIC FACTOR FOR DIAGNOSTIC AND THERAPEUTIC USE

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Lack of intrinsic factor, which occurs mainly in elderly people, leads to cobalamin malabsorption and eventually to cobalamin deficiency. In people who are well, gastric intrinsic

sic factor binds to cobalamin (vitamin B12) from the food, and the intrinsic factor-cobalamin complex is absorbed by the small intestine by binding to the intestinal receptor cubilin. Until now, the only human source of intrinsic factor has been from human gastric juice. However, it has recently been shown that recombinant human intrinsic factor produced in transgenic plants has the exact same binding profile as does native human intrinsic factor (Fedosov *et al.*, 2003). Both the native and recombinant proteins are able to discriminate between cobalamin and its derivatives like cobinamide. Likewise, the native protein as well as the recombinant protein will bind to the cubilin receptor only in complex with cobalamin. We are now capable of producing high-quality recombinant human intrinsic factor. The protein is produced according to GMP standards, and more than 80 per cent of the protein is active. Because our intrinsic factor is produced in plants it is free of human pathogens and contains absolutely no traces of other cobalamin-binding proteins like haptocorrin or transcobalamin. This makes it possible to improve research within the cobalamin field and diagnosis like the Schilling's test, the detection of serum cobalamin levels, and the presence of antibodies against intrinsic factor in serum from patients. For the first time, a large-scale production will make it possible to re-establish the natural cobalamin uptake in that large fraction of elderly patients who lack intrinsic factor.

**P055**

**THE RELATION BETWEEN HOMOCYSTEINE LEVELS AND DEVELOPMENT OF ALZHEIMER DISEASE IN MILD COGNITIVE IMPAIRMENT (MCI) PATIENTS**

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*Background.* Low levels of vitamin B12 in combination with folate deficiency have been shown to cause cognitive disturbances in elderly persons. Homocysteine levels will rise when there is a lack of the methyl donor folate and/or the co-enzyme cobalamin in the methionine cycle. A disturbed one-carbon metabolism may lead to the development of several forms of dementia.

*Aims.* The aim of this study was to investigate over a three-year period the connection between homocysteine levels and development of Alzheimers disease in patients with Mild Cognitive Impairment.

*Methods.* Homocysteine was analysed in 68 men and 68 women. Age, sex, cobalamin, folate, creatinine, thyroid profiles as well as results of Mini-Mental State Examination at the first visit to the memory investigation unit of a Geriatric Department were recorded from patient journals.

*Results.* The total numbers of persons who converted to Alzheimers disease within a period of three years from initial investigation with baseline homocysteine sampling was 12 of 46 (26%) males, and 18 of 50 women (36%). The total percentage of men and women converting to Alzheimers disease was 31%. Thirty-three percent of men with Hcy-levels >20 µmol/L converted to Alzheimers disease. The corresponding figure for men with Hcy-levels 20-17 µmol/L was 50%, whereas none of the 18 men with Hcy-levels <17 µmol/L converted to Alzheimers disease. These differences were statistically significant. There was also a statistically significant difference between the percentage of women with Hcy-levels >16µmol/l who converted to Alzheimers disease (45%) as compared to those with Hcy-levels <16 µmol/L who converted (21%).

*Conclusions.* These findings are inconsistent with the

results of other studies showing a positive correlation with hyperhomocysteinemia and occurrence of AD. However, our findings tentatively suggest a possible protective effect of low/normal Hcy levels on dementia conversion in MCI patients.

**P056**

**PLASMA TOTAL HOMOCYSTEINE AND COGNITION IN THE ELDERLY. THE HORDALAND HOMOCYSTEINE STUDY**

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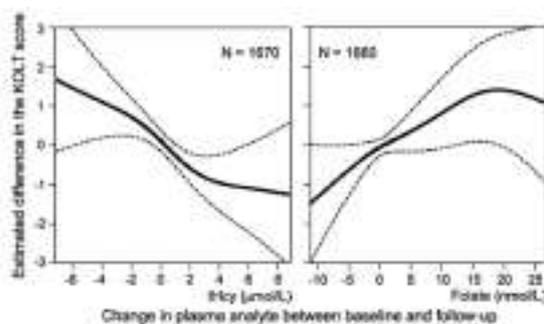
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*Background.* Moderately elevated plasma total homocysteine (tHcy) is associated with cognitive impairment.

*Aims.* To examine the relation between tHcy, folate, vitamin B12 and cognitive function in elderly community dwelling subjects.

*Methods.* A population-based study from Norway was conducted in 1992-1993 among subjects born in 1925-1952. The subjects were reinvestigated in 1997-1999. Blood samples for tHcy, folate and vitamin B12 were collected on both occasions. At follow-up, cognitive performance was investigated in 2189 elderly (70-72 years). The results using the Kendrick Object Learning Test (KOLT), a measure of memory performance, are reported. A KOLT score <25 was defined as cognitive deficit.

**Figure 1. The KOLT score and changes in tHcy and folate over time. Adjusted for gender, apolipoprotein E Å4 alleles, education, history of cardiovascular disease/hypertension, depression and baseline tHcy or folate. The reference value for KOLT is the approximate value associated with no change over the 6-year period in tHcy or folate levels, respectively. The solid lines are the estimated dose-response curves, and the dashed lines are the 95% confidence intervals.**



*Results.* Subjects with cognitive deficit had higher follow-up tHcy and lower folate concentrations compared to those without: 12.6 (95% CI: 12.1, 13.1) micromol/L versus 11.5 (11.3, 11.6) µmol/L, *p*<0.001 for tHcy and 6.7 (6.2, 7.1) nmol/L versus 7.6 (7.5, 7.8) nmol/L, *p*<0.001 for folate. After adjustments for several potential confounders, the risk of cognitive deficit increased according to quintiles of tHcy; OR for tHcy quintiles: 1.00, 1.17, 0.98, 1.40, 1.87 (*p*-trend=0.007) at baseline, and 1.00, 1.05, 1.70, 1.66, 2.34 (*p*-trend<0.001) at follow-up. An inverse association of cognitive deficit with quintiles of folate or vitamin B12 was found, but was significant

only for baseline values [OR for folate quintiles: 1.64, 1.51, 1.07, 0.96, 1.00 ( $p$ -trend=0.006), and for vitamin B12 quintiles: 1.63, 1.26, 1.30, 1.08, 1.00 ( $p$ -trend=0.042)]. A decline in tHcy, or a rise in folate concentrations, over six years was associated with higher KOLT score, while a rise in tHcy, or a decline in folate, was associated with a lower score (Figure).

**Conclusions.** Elevated plasma tHcy is an independent risk factor for cognitive deficit both cross-sectionally and prospectively. An important observation is that a favorable change in folate or tHcy status over a 6-year period is associated with better cognitive performance.

#### P056a

##### EFFECT OF DAILY ORAL VITAMIN B12 AND VITAMIN B12/FOLATE SUPPLEMENTATION ON COGNITIVE PERFORMANCE IN ELDERLY PEOPLE WITH VITAMIN B12 DEFICIENCY: A RANDOMIZED PLACEBO CONTROLLED TRIAL

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**Background.** Cognitive impairment is associated with vitamin B12 deficiency in both healthy and cognitively impaired elderly people. However, results of intervention trials on vitamin B12 supplementation and cognitive performance are scarce and inconclusive.

**Aims.** To investigate whether daily oral supplementation during 6 months with 1,000 µg vitamin B12 or 1,000 µg vitamin B12 combined with 400 µg folate improves cognitive performance in 195 people over 70 years with vitamin B12 deficiency.

**Methods.** In this randomized, double-blind, parallel group, placebo controlled trial, a stratified randomisation procedure was used to ensure a balanced distribution of participants with respect to methylmalonic acid (MMA), age, sex, and MMSE at baseline. Vitamin B12 status, including methylmalonic acid (MMA), homocysteine (Hcy) and transcobalamin II (holoTC), was measured at baseline, and at 3 and 6 months of intervention, while cognitive performance was measured at baseline and at 6 months of intervention. General cognitive performance was assessed by the MMSE. More specifically, attention was assessed by Digit Span Forward; speed by Finger Tapping and Trail Making A; memory by the 15 Word Learning Test, Complex Figure of Rey, and Digit Span Backward; executive function by the Motor Planning Task, Trail Making (C/A), and Stroop; and fluid intelligence by the WAIS, Word Fluency and Raven.

**Results.** Average age of the study population was 82±5 years, 24% was male, and 57% were free-living elderly. Supplementation with vitamin B12 and vitamin B12/folate was associated with mean reductions in plasma MMA concentrations of 42% and 36%, while vitamin B12 concentration increased with 198% and 230%, respectively. At baseline, cognitive impairment appeared to be most pronounced in the cognitive domain memory. The effects of supplementation on cognitive performance will be presented during the conference.

#### P057

##### HOMOCYSTEINE, METHYLENETETRAHYDROFOLATE REDUCTASE AND RISK OF SCHIZOPHRENIA

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**Background.** Evidence for an involvement of aberrant homocysteine metabolism in the aetiology of schizophrenia is limited and controversial.

**Aims.** We performed a case-control study to quantify the risk of schizophrenia in the presence of elevated homocysteine concentrations or homozygosity for the 677C>T polymorphism (677TT) in the methylenetetrahydrofolate reductase (MTHFR) gene in subjects of Dutch ancestry. In addition, a meta-analysis was carried out with published data including the present study.

**Methods.** 677C>T MTHFR was determined in 254 patients and 414 healthy controls. Plasma homocysteine concentrations were measured in 62 patients with schizophrenia and 432 control subjects. The meta-analysis included 7 studies (721 cases and 1865 controls) which examined the association of homocysteine levels with schizophrenia, and 7 studies (1373 cases and 1722 controls) with available data on the MTHFR 677C>T genotypes and schizophrenia.

**Results.** We calculated an increased risk for schizophrenia in the fourth and third quartile versus the lowest quartile [odds ratio (OR)=3.3; 95% confidence interval (CI): 1.2 to 9.2, and OR=3.1; 95% CI: 1.2 to 8.0 respectively]. A significant dose-response relation of increasing homocysteine levels and increasing risk for schizophrenia was observed ( $p$ =0.036). The 677TT genotype was associated with an odds ratio of 1.6 [95% CI: 0.96 to 2.8] of having schizophrenia. When all available data were combined a 5 micromol/L higher measured homocysteine level was associated with a 93% (95% CI: 64 to 127) higher risk of schizophrenia. The TT genotype was associated with a 54% (95% CI: 23 to 93) higher risk of schizophrenia compared with the CC genotype in the meta-analysis.

**Conclusions.** Elevated homocysteine levels and the MTHFR 677TT genotype are associated with an increased risk for schizophrenia. These observations support a causal relation between disturbed homocysteine metabolism and schizophrenia.

#### P058

##### CLINICAL EFFECTS OF CO-ADMINISTERING N-ACETYLCYSTEINE, VITAMIN B12 AND FOLATE IN COGNITIVELY IMPAIRED HYPERHOMOCYSTEINAEMIC PATIENTS

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**Background.** Evidence accumulates for a relationship between homocysteine and cognition in the elderly. Plasma homocysteine is elevated in histopathologically confirmed Alzheimer's disease (AD), and patients with higher levels have greater radiological evidence of disease progression. Homocysteine levels predict cognitive decline in healthy elderly and hyperhomocysteinaemia is now a recognized risk factor for dementia, including AD. Potential mechanisms by which it exerts these effects include a direct toxicity on glu-

## Homocysteine Metabolism

tamate neurotransmission and cerebrovascular endothelium and an indirect inhibition of transmethylation reactions. Plasma levels are largely determined by vitamin B12 and folate status. However, the methionine synthase reaction is extremely sensitive to oxidative stress - a key feature of the pathological cascades in both AD and vascular dementia. Antioxidant therapy might be required in addition to B vitamins for optimal homocysteine reduction and maximum clinical benefit in these disorders.

**Aims.** To evaluate the clinical effects of co-administering N-acetylcysteine (NAC), a glutathione precursor and potent antioxidant, together with standard B vitamin supplements in cognitively impaired hyperhomocysteinaemic patients in a General Practice setting.

**Methods.** We studied ten individuals consecutively presenting to their GP with objective evidence of cognitive impairment and elevated plasma homocysteine. Fasting homocysteine levels were determined by HPLC and cognitive function assessed by MMSE and ADAS-Cog scores.

**Results.** Correcting hyperhomocysteinaemia by B vitamin supplementation alone did not improve cognitive scores. However, the addition of NAC resulted in significant clinical improvement. At the oral dosages used (600 mg -1200 mg daily) NAC was not associated with any significant adverse reactions.

**Conclusions.** This small case-series suggests that NAC is efficacious in combination with B vitamins in improving cognitive function. There is one previous report of NAC treatment alone in AD patients. A randomized controlled clinical trial is now underway to formally evaluate this novel synergistic approach in hyperhomocysteinaemic patients with mild cognitive impairment.

### P059

#### LOCALIZATION OF ENZYMES THAT REGULATE FOLATE AND HOMOCYSTEINE METABOLISM IN THE MURINE BRAIN

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Impairments in folate and/or homocysteine metabolism are associated both with developmental anomalies and an increased risk for negative cognitive outcomes in aging populations. Despite these known associations, little is known regarding the regulation of folate metabolism, including homocysteine remethylation, in the central nervous system. To address this paucity of information, we examined the expression of several enzymes that regulate folate-based one-carbon metabolism in the mouse brain. Expression of cytoplasmic serine hydroxymethyl transferase (cSHMT), methenyl tetrahydrofolate synthetase (MTHFS), and C1 tetrahydrofolate synthase (C1 synthase) was characterized in both young and adult Bl/6 mice to address changes in expression that occur across the lifespan. In situ hybridization and R-galactosidase staining of transgenic animals was used to determine the presence of mRNA transcripts of these genes in the brain, and immunohistochemistry was used to verify the presence of the corresponding proteins. Expression of cSHMT and C1 synthase was highly restricted, whereas expression of MTHFS was widespread in the mouse brain. Specifically, expression of cSHMT and C1 synthase was observed mostly along the ventricles, in the hippocampus, and in the cerebellum. Further, preliminary comparison of expression patterns between young and aged animals revealed a decrease in expression of cSHMT and C1 synthase with age. These findings will be discussed in the

context of known associations between elevated homocysteine levels and cognitive outcomes, as well as the broader role of one-carbon metabolism in the adult brain.

### P060

#### EFFECT OF THE B-VITAMINS ON COGNITIVE FUNCTION IN ELDERLY PEOPLE WITH MILD COGNITIVE DYSFUNCTION

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**Background.** Hyperhomocysteinaemia have been related to cognitive dysfunction in older age.

**Aims.** We conducted a double-blind placebo-controlled study to investigate the effect of vitamin treatment on cognition in elderly subjects.

**Methods.** The study included 71 subjects (mean age/SD=81/15) with GFR>35 ml/min and MMSE score (Mini-Mental State Examination test)>15. Subjects were allocated to receive placebo (n=35) or vitamins (n=36). A daily s.c. injection of placebo or vitamins (1mg B12, 5 mg B6, 1.1 mg folate) were administered for 3 weeks. This was followed by an oral treatment for additional 3 weeks. The MMSE and Structured Interview for the Diagnosis of Dementia of the Alzheimer Type (SIDAM) tests were assessed before and at the end of the study.

**Results.** At baseline, higher concentrations of methylmalonic acid (MMA) were associated with lower intellectual abilities ( $p=0.011$ ). Serum concentrations of MMA were lowered only in the vitamin group ( $p<0.001$ ). Concentrations of homocysteine were also lowered in the vitamin group (mean = 9.6 vs. 17.0  $\mu\text{mol/L}$ ;  $p<0.001$ ) but not in the placebo group (mean =17.2 vs. 16.1  $\mu\text{mol/L}$ ;  $p>0.05$ ). Cognitive function improved in both groups, however, only the vitamin group showed significant improvements in SIDAM-scores after the treatment (mean= 43 vs. 38;  $p=0.011$  and 44 vs. 40;  $p=0.196$  in the placebo group). A marked improvement was also observed in intellectual capacities and higher cortical functions in the vitamin, but not in the placebo group.

**Conclusions.** We conclude that therapeutic doses of the vitamins B may improve some cognitive functions in elderly people with a mild cognitive impairment.

### P061

#### HOMOCYSTEINE LEVELS IN PATIENTS WITH ALZHEIMER'S DISEASE ARE INFLUENCED BY THE GLUTATHIONE S-TRANSFERASE OMEGA-1 (GSTO1) GENE

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**Background.** A substantial body of literature confirms an association between elevated blood levels of homocysteine and cognitive dysfunction, including Alzheimer's disease (AD). Oxidative stress is a risk factor for AD. Elevated homocysteine levels might partially reflect redox status; its remethylation to methionine is coordinated by the redox-sensitive enzyme methionine synthase. Glutathione S-transferase omega-1 (GSTO1) is protective against oxidative stress, and the polymorphism Ala140Asp modifies the age-of-onset of AD.

**Aims.** To investigate whether the GSTO1 Ala140Asp polymorphism is related to homocysteine levels in AD patients.

**Methods.** Plasma homocysteine levels and the GSTO1 polymorphism Ala140Asp were analysed in 244 consecu-

tive patients with clinically diagnosed AD.

**Results.** Homocysteine levels differed significantly between the three genotypes ( $p=0.002$ ) analysis of variance, Durbin-Watson D Statistic. The levels were  $11.8\pm 3.6$   $\mu\text{mol/L}$  in patients with the Ala/Ala genotype ( $n=118$ ),  $13.5\pm 5.0$   $\mu\text{mol/L}$  in the Ala/Asp group ( $n=105$ ), and  $14.1\pm 6.0$   $\mu\text{mol/L}$  in patients with the Asp/Asp genotype ( $n=21$ ). Carriers of at least one Asp allele showed significantly higher plasma homocysteine levels compared to non-carriers ( $p=0.002$ ) two-sample t-test.

**Conclusions.** The association between homocysteine levels and this GSTO1 polymorphism supports the suggestion that increased homocysteine in AD patients may be a consequence of oxidative stress.

## P062

### FOLATE-DEFICIENCY-INDUCED COGNITIVE DEFICITS IN RAT ARE AMELIORATED BY METHIONINE AND ARE UNRELATED TO HOMOCYSTEINEMIA

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**Background.** Homocysteinemia is associated with age-related cognitive decline and dementia in humans but whether the cognitive deficits are due to toxic effects of homocysteine or to the primary metabolic imbalance is unclear. Dietary folate deficiency results in a fasting homocysteinemia due to inhibition of methionine synthesis from homocysteine. Excess dietary methionine results in a transient post-prandial homocysteinemia due to overproduction of homocysteine.

**Aims.** To determine the impact of dietary imbalances in folate and/or methionine and their resultant homocysteinemias on behavioral outcomes in rats.

**Methods.** Male 4 week-old Sprague Dawley rats were fed experimental diets containing either 0.2 (control) or 8 mg folic acid per kg diet; and/or 0.35% (control), 1.35% or 2.35% L-methionine. At 9 weeks, rats underwent behavioral testing using the RotaRod and wire-hang tests of psychomotor function and the Morris Water Maze test of visuospatial memory and learning. Rats had access to food during testing. At 10 weeks tissues were harvested for hematological, biochemical and histopathological analysis.

**Results.** Folate deficiency resulted in a persistent (fasting) homocysteinemia of approximately 30 micromolar. Excess methionine intake produced a postprandial rise in blood homocysteine to concentrations of approximately 80-120 micromolar which returned to normal (approximately 5 micromolar) within 24 hours. A behavioral deficit in Morris Water Maze performance was only observed in rats fed the folate deficient diet. This behavioral deficit was abolished by the addition of methionine to the folate deficient diets, even though the additional methionine did not diminish the homocysteinemia. Folate deficient animals were not anemic, whereas excess methionine produced a slight decrease in red blood cell counts and a significant increase in mean corpuscular volume. Gross neurodegeneration was not observed for any diet.

**Conclusions.** Folate deficiency rather than homocysteinemia is associated with a behavioral impairment that is likely mediated by a brain requirement for methionine.

## P063

### CHRONIC B12 DEFICIENCY INDUCES HOMOCYSTEINEMIA AND ANEMIA WITHOUT NEUROCOGNITIVE DEFICITS IN RAT

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**Background.** Homocysteinemia is associated with age-related cognitive decline and dementia in humans but whether the cognitive deficits are due to toxic effects of homocysteine or to the primary metabolic imbalance is unclear. Folate and vitamin B12 deficiency result in a fasting homocysteinemia. In humans chronic vitamin-B12 deficiency can cause demyelinating neuropathy or dementia but this has not been shown in rat. In contrast, we find that short-term folate deficiency is sufficient to induce both homocysteinemia and behavioral deficits in rat.

**Aims.** To determine the behavioral and neurological impact of chronic B12-deficiency and its resultant homocysteinemia in rat.

**Methods.** Male 4 week-old Sprague Dawley rats were fed experimental diets containing either 0 or 25 micrograms of vitamin B12 (cyanocobalamin) per kg diet. Tissues were harvested for hematological, biochemical and histopathological analysis after rats had consumed the diets for either 6, 12, 18 or 43 weeks. One week prior to this they underwent psychomotor testing, the radiant-heat tail flick test for evidence of peripheral neuropathy, and the Morris Water Maze test of visuospatial learning and memory.

**Results.** In rats fed the B12 deficient diets, plasma B12 levels fell below 150 pmol/L within 6 weeks and were undetectable by week 12. Fasting plasma homocysteine concentrations attained a level of approximately 10 micromolar by week 6 and 25 micromolar by week 18. Anemia was not observed at 6, 12 or 18 weeks, but developed by week 43. Nevertheless, despite persistent B12-deficiency, homocysteinemia, and even anemia at week 43, behavioral deficits were not observed in these rats at any time.

**Conclusions.** In contrast to folate-deficient homocysteinemia, chronic B12-deficiency fails to produce behavioral deficits in rat. This suggests that the primary metabolic disturbance of homocysteine metabolism is central to the association of homocysteinemia and cognitive dysfunction.

## P064

### CEREBROSPINAL FLUID HOMOCYSTEINE AND HYDRODYNAMICS IN CHRONIC HYDROCEPHALUS

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**Background and Aims.** Homocysteine seems to have a direct neurotoxic effect and could increase the vulnerability to excitotoxic and oxidative injury. Cerebrospinal fluid (CSF) may play a role in the clearance or secretion of Homocysteine. Hydrocephalus, a partially reversible cognitive impairment, is mainly due to the ill-circulation of the CSF. The aim of this study is to gauge homocysteine and other thiols in the CSF of hydrocephalic patients, and to correlate these biological parameters with hydrodynamic patterns.

**Methods.** Forty-three patients (24 females, 19 males, mean

age: 71,6 years) were included. All patients were suspected of chronic hydrocephalus (gait alteration, urinary incontinence and cognitive decline) and enlargement of the ventricles on brain imaging. To comfort the diagnosis, we performed in clinical routine CSF hydrodynamic tests via lumbar infusion according to Czosnyka's technique. A lumbar needle was inserted. A 2 mL sample of CSF was withdrawn for biochemical analysis. Subsequently, we measured the CSF hydrodynamics: pressure and pulse amplitude of the CSF during baseline and plateau, and calculation of the resistance to CSF outflow. Homocysteine (Hcy), Cysteine (Cys), Cysteinyl-Glycine (Cys-Gly) and Glutathione (G-SH) were measured in CSF by capillary eletrophoresis and laser induced fluorescence detection.

**Results.** In the CSF of the patients, the mean± SD values of the various thiols are: Hcy 0.24±0.05 μmol/L, Cys 3.84±1.26 μmol/L, Cys-Gly 3.33±0.92 μmol/L and G-SH 0.5±0.45 μmol/L. Cys and Cys-Gly were positively correlated ( $r=0.51$   $p<0.01$ ). Baseline CSF pressure and resistance to CSF outflow were not correlated with the thiol levels. However, the baseline pulse amplitude was weakly but significantly correlated to Hcy level ( $r=0.36$   $p=0.02$ ).

**Conclusions.** Our preliminary results suggest that in hydrocephalus, hydrodynamics is not related to the level of thiols. Our initial hypothesis is not supported by our data. However, our results raise the interest of transsulfuration pathway of the thiol metabolism in the CSF.

**P065**

**HIGH LEVEL OF PLASMA HOMOCYSTEINE IN CHILDREN WITH AUTISM**

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Autism is a behaviorally defined disorder of unknown etiology that is thought to be influenced by genetic and environmental factors. High levels of homocysteine and oxidative stress are generally associated with neuropsychiatric disorders. Children with autism have not been investigated from this point of view. The purpose of this study was to compare the level of homocysteine and other biomarkers in children with autism with corresponding values in age-matched healthy children. We measured total homocysteine (tHcy), vitamin B12, paraoxonase and arylesterase activities of PON1 (human paraoxonase 1) in plasma and glutation peroxidase (GPX) activity in erythrocytes from 21 children: 12 with autism (age 8.29, SD=2.76) and 9 controls (8.33, SD=1.82). Total Hcy was measured by HPLC with pre-column derivatization and fluorescence detection. Electrochemiluminescence immunoassay was used for vitamin B12. Paraoxonase (NaCl stimulated) and arylesterase activities of PON1 were determined with spectrophotometric methods by using paraoxon and phenylacetate as substrates. GPXase was assayed spectrophotometrically in a coupled reaction with glutation reductase in the presence of NADPH. We found statistically significant differences in tHcy level and in arylesterase activity of PON1 between the children with autism and the control group: 9.83 (SD=2.75) micromoles vs. 7.51 (SD=0.93) micromoles ( $p=0.01$ ) and 72.57 (SD=11.73) units vs. 81.83 (SD=7.39) units ( $p=0.005$ ). There were no other statistically significant differences between the two groups in other evaluated biomarkers, but in children with autism vitamin B12 level was suboptimal. In conclusion, our study shows that in children with autism there are high levels of tHcy and low levels of arylesterase activity of PON1 associated with suboptimal levels of vitamin B12.

**P066**

**THE ASSOCIATIONS OF AGE, PRIOR CARDIOVASCULAR MORBIDITY AND TOTAL PLASMA HOMOCYSTEINE WITH RATES OF CHANGE OF BRAIN VOLUME IN OLDER ADULTS**

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**Background.** Age, vascular risk factors and Alzheimer's disease (AD) have been associated with cross-sectional differences in MRI brain volumes.

**Aims.** To investigate the associations among cardiovascular risk, plasma total homocysteine (tHcy) and the rates of change in whole brain (WB) volume and ventricular (V) volume per year.

**Methods.** 145 'stroke-free', non-demented (60-90 years) community-dwelling volunteers were followed in a longitudinal cohort study. MRI-derived percentage WB and V volume change per year, baseline plasma total homocysteine (tHcy), prior cardiovascular morbidity group (no CVD, CVD) and baseline cardiovascular (CV) factors (systolic blood pressure, smoking, diabetes mellitus, LDL/HDL ratio, ApoE genotype, aspirin and anti-hypertensive medication usage) were assessed.

**Results.** Median percentage rates of change of volume per year were 0.66 for WB loss and 4.1 for V gain. Age-adjusted baseline V volumes ( $\rho=0.16$ ,  $p=0.058$ ) were more highly correlated with tHcy level than with WB volume ( $\rho=-0.13$ ,  $p=0.13$ ). Age, CVD group, the interaction of Age x CVD ( $t=3.6$ ,  $p<0.001$ ), but not tHcy, were strongly positively associated with the rate of WB volume loss per year. The interaction of tHcy x CVD, and not tHcy, was positively associated with rates of V volume gain per year ( $t=2.0$ ,  $p<0.05$ ), and WB volume loss per year ( $t=1.8$ ,  $p=0.07$ ), especially in men. Other CV factors did not confound nor add to these associations.

**Conclusions.** The interactions of age and tHcy level with prior cardiovascular morbidity are significantly associated with the rates of change of brain volume in older adults. These findings may offer insight into the pathophysiology of brain atrophy with increasing age and tHcy levels.

**P067**

**LIPOTROPIC ACTION OF BETAINE IN A MOUSE MODEL OF HYPERHOMOCYSTEINEMIA DUE TO A DEFICIENCY OF METHYLENETETRAHYDROFOLATE REDUCTASE**

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To investigate the lipotropic action of betaine on plasma lipoproteins and lipid accumulation in tissues, we supplemented adult mice either wild type or heterozygous for a disruption of the methylenetetrahydrofolate reductase gene with betaine over a one-year period and compared them with control groups on regular mouse chow. Outcome measures were aortic and liver morphology and plasma homocysteine and lipoproteins after one year. In a co-study we investigated the short-term effects of a two weeks course

of supplemental betaine on plasma lipoproteins in female mice either wild-type or heterozygous for a disruption of the MTHFR gene. Mice of either genotype showed lower plasma total homocysteine after long-term supplementation with betaine. Heterozygous MTHFR mice and wild-type mice also had lower plasma triglycerides and higher HDL-cholesterol both after short and long-term betaine supplementation. Lipid accumulation in liver and aorta wall showed a trend towards less deposition in wild type versus heterozygous mice and in betaine-supplemented versus non-supplemented mice. We conclude that betaine has a lipotropic effect in our model and that this effect is associated with lowering of plasma homocysteine. Heterozygous MTHFR deficiency in mice appears to be associated with lipid deposition in liver and aorta and thus may promote the development of atherosclerosis.

## P068

### SEQUENCE ANALYSIS OF THE DIHYDROFOLATE REDUCTASE GENE

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**Background.** Hyperhomocysteinemia is an independent risk factor for various diseases including arterial vascular disease and venous thrombosis. The enzyme dihydrofolate reductase (DHFR) catalyzes the reduction of folate into dihydrofolate (DHF) and DHF into tetrahydrofolate. Inhibition of DHFR by the antifolate methotrexate results in elevated plasma total homocysteine (tHcy).

**Aims.** In order to identify genetic variants that may affect tHcy and disease risk, the DHFR gene of 20 recurrent venous thrombosis (RVT) patients was sequenced.

**Methods.** Selection criteria for sequence analysis: Six RVT cases with low folate (<10th percentile=9.0 nM), nine cases with high tHcy (>90th percentile=15.5 mM) and another 5 patients were selected randomly. No MTHFR 677TT cases were included nor cases with a vitamin B12 <10th percentile (125 pM). Each exon was amplified using intron-based oligonucleotides. In addition, the 5' and 3' untranslated regions were sequenced.

**Results.** The coding region of the DHFR did not harbour any variation in our selected cases. We identified a 9bp repeat in the 5' untranslated/promoter region of the DHFR and cloned the alleles containing the 3x, 6x and 7x repeat although other repeat sizes may also be present. The repeat is currently genotyped by means of Genescan analysis and the impact of this variant on tHcy, plasma folate and disease risk will be evaluated in a group of recurrent venous thrombosis patients and population-based controls. In addition, the 19bp deletion/insertion in intron 1 of DHFR will be assessed for its effect on tHcy, plasma folate and disease risk in the same populations.

**Conclusions.** We discovered a 9bp repeat in the promoter region of DHFR. DHFR is essential for transmethylation and DNA synthesis and shares the promoter with the DNA mismatch repair gene hMSH3. Altered gene expression or mRNA stability by virtue of a repeat expansion may have important implications in these processes.

## P069

### ELEVATED HOMOCYSTEINE LEVELS IN FIRST EPISODE PSYCHOSIS. AN ANALYSIS OF HOMOCYSTEINE LEVELS IN A COHORT OF FIRST EPISODE PSYCHOSIS PATIENTS AND AN AGE AND SEX MATCHED HEALTHY CONTROL GROUP

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**Background.** Homocysteine and folate metabolism is of renewed interest in schizophrenia and psychosis research. Elevated homocysteine in people with schizophrenia, particularly in young males who tend to have a more adverse outcome, led us to examine homocysteine levels in a first episode psychosis cohort.

**Methods.** Participants were recruited from the Early Psychosis Prevention and Intervention Centre (EPPIC), a sub-program of ORYGEN Youth Health. EPPIC serves a geographical catchment area of 800,000 and provides a first episode psychosis service for young people aged between 15 and 25. Participants consented to a randomised placebo controlled trial investigating the homocysteine lowering effects of a B-complex Vitamin on cognition and symptomatology, as add on to existing antipsychotic medication. Normal healthy controls, from the same geographical catchment area with no family history of psychosis were recruited as an age and sex matched comparison group. Confounding variables for homocysteine levels are being assessed through the use of diet questionnaires, substance misuse measures and genetic analysis for MTHFR polymorphisms.

**Results.** So far 33 first episode psychosis patients and 25 controls have been recruited. Analysis shows Homocysteine is significantly elevated in first episode psychosis patients compared to an age and sex matched control group.

**Conclusions.** These findings add to the growing literature on homocysteine and psychosis. Elevated homocysteine indicates a metabolic disturbance of one-carbon metabolism which may contribute to the pathophysiology of psychosis. Homocysteine is neurotoxic, promotes apoptosis and has been linked to cognitive decline. Lowering homocysteine levels through the use of Folate, B12 and B6 may have beneficial effects on cognition and symptomatology in first episode psychosis.

## P070

### RELATIVE EFFECTS OF B VITAMIN DEPLETION ON PLASMA HOMOCYSTEINE

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Homocysteine (hcy) is an independent risk factor for cardiovascular disease. Folate, vitamin B12, pyridoxine and riboflavin are integral co-factors in hcy metabolism. Various studies have examined the effects of B vitamins on hcy, but none have examined the independent roles of depletion of each B vitamin alone. The evidence is uncertain therefore as to the relative effects of low levels of the four B vitamins on hcy. The aim of this study is to investigate the impact of marginal deficiency of folate and pyridoxine on hcy while controlling for intakes of all other B vitamins. Low folate (0.55 micrograms per day) and low pyridoxine (0.36 milligrams per day) diets were designed (7-day cycles). Volunteers were randomised to one of the two depletion diet groups: low folate (6 weeks); low pyridoxine (4 weeks) or control group (6 weeks; n=5, 5, 6 in each group, respective-

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ly). Volunteers in the control group consumed their normal diet and a multivitamin containing all vitamins and minerals. Volunteers on each of the low B vitamin diets consumed a deplete diet and in addition a multivitamin/multimineral tablet (devoid of the respective B vitamin). Plasma hcy was measured weekly. Changes in hcy in response to depletion were compared between low-folate, low-pyridoxine and control groups. Responses among the low-pyridoxine group were not significantly different from the control group, whereas among the low-folate group, responses were significantly different from both low-pyridoxine and control groups ( $p=0.008$ ; one-way ANOVA & Tukey post hoc). Following depletion to marginal deficiency of the B vitamins folate and pyridoxine, folate depletion induces a significant elevation in hcy, whereas pyridoxine depletion has no effect.

**Table 1. Pre and post tHcy concentrations in deplete and control groups.**

	Folate (n=5)	Pyridoxine (n=5)	Control (n=6)
Pre tHcy (micromol/L)	10.2 (2.3)	9.4 (1.7)	11.3 (1.1)
Post tHcy (micromol/L)	14.7 (2.0)	6.8 (1.3)	9.3 (0.9)
% change	60 (21)	-24 (12)	-13 (13)

All values are mean (SEM).

## P071

### THE EFFECTS OF SPREADS ENRICHED WITH B-VITAMINS ON HOMOCYSTEINE AND HS-CRP IN HEALTHY SUBJECTS

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**Objective.** To determine the effect of a B-vitamin enriched spread, with or without plant sterols, on the concentration of homocysteine and high sensitivity C-reactive protein (hs-CRP).

**Design.** A randomized double-blind placebo-controlled parallel trial including 123 healthy male (n=50) and female (n=73) volunteers (mean age 57, range: 33-74 years). Volunteers consumed a daily dose of 20g spread for a period of 12 weeks. The spreads were low-fat margarine's (~40%), containing per 20 g: 200 µg folic acid, 1 mg vitamin B6 and 1 µg vitamin B12 (B), 200 µg folic acid, 1 mg of vitamin B6 and 1 µg vitamin B12 and 2.25 g plant sterols (BS) or no B-vitamins or sterols (placebo, P). Blood was drawn for measurement of outcome variables at baseline and at 12 weeks.

**Results.** Mean plasma homocysteine concentration at baseline was 10.0 (B), 10.1 (BS) and 10.0 (P) µmol/L, and did not differ between the groups. After 12 weeks the plasma homocysteine concentrations were: 8.0 (B), 7.8 (BS) and 10.7 (P) µmol/L. The relative decrease in the groups that received the B-vitamin enriched spreads as compared to placebo were respectively -26.1% (B) and -27.9% (BS) ( $p<0.05$ ). Hs-CRP concentrations at baseline and 12 weeks were available for 80 subjects (37 males and 43 females). Mean hs-CRP concentrations at baseline were: 2.0 (B), 2.1 (BS) and 1.7 (P) mg/L, and did not differ between the groups. Within each intervention group the hs-CRP concentration decreased after 12 weeks (statistically significant for group B as compared to baseline: -0.3 mg/L,  $p=0.007$ ), but compared to placebo, the changes were not statistically significant. Potential confounding factors such as age, smoking and BMI did not change the results for hs-CRP.

**Conclusions.** Spreads enriched with B-vitamins with or without plant sterols, significantly decreases the homocys-

teine concentration with ~27%, but do not influence the concentration of hs-CRP, a marker of inflammation.

## P072

### VERY LONG CHAIN OMEGA-3 FATTY ACIDS LOWER THE PLASMA HOMOCYSTEINE CONCENTRATION IN ADULTS AT INCREASED RISK OF CVD A META-ANALYSIS

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**Background.** Elevated total plasma homocysteine concentrations (tHcy) are associated with an increased risk of cardiovascular diseases (CVD). Some intervention trials with very long chain (VLC) omega-3 fatty acids suggest that these fatty acids may contribute to a lower tHcy concentration.

**Objective.** To verify whether VLC omega-3 fatty acids have a tHcy-lowering effect in a meta-analysis.

**Design.** Of nine identified intervention trials, four met our quality criteria. The individual data of the subjects of these trials (n=408) were pooled and analyzed. All subjects were at increased risk of CVD either due to hyperlipidemia or a history of CVD.

**Results.** Baseline tHcy concentrations in the placebo and the intervention group were similar (13.6 vs. 13.9 µmol/L,  $p=0.6$ ). After intervention with VLC omega-3 fatty acids the tHcy concentration changed with -0.7 vs. -0.2 µmol/L in the placebo group ( $p=0.2$ ). Further analysis indicated that the change depended on baseline tHcy concentration and study. In addition, the intervention effect was different in adults than in elderly, thus analyses were stratified for age ("65 vs. >65 years). Stratified analyses were based on data of 352 subjects, due to the absence of individual age data for one trial. In adults, the adjusted change in tHcy after intervention was -1.2 vs. -0.4 µmol/L in the placebo group ( $p=0.07$ ). This corresponded to relative changes of -4.4% vs. 1.5% ( $p=0.03$ ). In elderly no effect of the intervention was observed.

**Conclusions.** Increased intakes of VLC omega-3 fatty acids, in a dose varying between 0.25-5 g/d, results in a lower tHcy concentration in adults "65 years. Several health authoritative bodies recommend a higher VLC omega-3 fatty acid intake in subjects at increased risk of CVD because of beneficial effects on heart health such as blood pressure and triglyceride lowering. Homocysteine-lowering could be added to the list of beneficial effects of VLC omega-3 fatty acids.

## P073

## EFFECT OF GAMMA-CYSTATHIONASE 1364G-T (S403I) GENOTYPES ON TOTAL PLASMA HOMOCYSTEINE

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**Background.** The total plasma homocysteine (tHcy) concentration, an independent risk factor for atherothrombosis, is determined by several environmental and genetic factors.

**Aims.** We investigating the effect of a common single nucleotide polymorphism in gamma-cystathionase 1364G-T (S403I) on plasma tHcy.

**Methods.** Clinical and biochemical data and the S403I gamma-cystathionase and C667T MTHFR genotypes were available from 486 healthy subjects (median 49 [16-100] years, 73% female) of Caucasian origin. Genotypes were determined by TaqMan Analyses. Exclusion criteria were creatinine >1.4 mg/dL and intake of vitamins and medications known to interfere with homocysteine metabolism.

**Results.** In this cohort of Austrian subjects, Genotype frequencies were 0.486, 0.426 and 0.090 for gamma-cystathionase 403S/S, 403 S/I and 403 I/I, respectively. Allele frequencies were 0.205 for MTHFR 677T and 0.303 for gamma-cystathionase 403I. Homozygosity (II) for 403 gamma-cystathionase was associated with 2.2  $\mu$ mol/L higher tHcy levels (14.0 vs. 11.9) as compared to SS/SI. Homozygosity (TT) for MTHFR 677 was associated with higher tHcy levels (15.7 vs. 14.0 [CT] and vs. 12.8 [CC]  $\mu$ mol/L). All values and study groups were age-adjusted. Excluding subjects with folate <6 ng/mL, TT+SS/SI vs. II+CC/CT differed significantly ( $p=0.027$ ) suggesting synergistic homocysteine-elevating effects of both homozygous genotypes. Conclusions The findings suggest a relevant determining effect of this common polymorphism in gamma-cystathionase 1364G(rT) (S403I) on plasma homocysteine levels in a healthy population.

## P074

## DETERMINANTS OF tHcy LEVELS IN BRAZILIAN MOTHER AND NEWBORNS

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The interaction between nutritional and genetic factors can impair homocysteine metabolism. The aim of this study was to evaluate the association between maternal and neonatal vitamin levels and the determinants of tHcy in mother and newborn. Genotypes for MTR A2756G and MTRR A66G polymorphisms were determined by PCR-FLRP. The levels of cobalamin, red blood cell (RBC) folate, serum folate (SF), total homocysteine (tHcy), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and SAM/SAH ratio were also determined in 369 pregnant women (37-42 weeks of gestational age) and their newborns. Three models of linear regression analysis with stepwise were analysed (Table). We conclude that maternal

serum folate and Cbl levels and maternal MTR 2756AA genotype may affect tHcy metabolism in Brazilian pregnant women and their neonates.

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Dependent variables N	Independent variables	B	P value	R <sup>2</sup> Partial
MODEL 1	1. Maternal SF*	-0.423	<0.001	19.11
Maternal tHcy*	2. MTR AG+GG (ref.)	0.000	1.000	
341	MTR AA	0.033	0.043	0.93
	1. Maternal SF*	-0.310	<0.001	13.97
MODEL 2	2. Maternal Cobalamin*	-0.150	0.011	3.02
Neonatal tHcy*	3. MTR AG+GG (ref.)	0.000	1.000	
269	MTR AA	0.044	0.011	1.77
	4. Maternal Creatinine *	0.190	0.043	1.22
MODEL 3	1. Neonatal Cobalamin*	-0.179	<0.001	7.58
Neonatal tHcy*	2. Neonatal SF*	-0.373	<0.001	5.99
269	3. Neonatal SAM/SAH*	-0.127	0.002	3.16

\*Log transformed variable. The regression models were adjusted by maternal age, ethnic group and parity. Model 1: Maternal variables as determinants of maternal tHcy. Independent variables - maternal RBC and serum concentrations of folate, Cbl, creatinine, SAM/SAH ratio, maternal MTR genotypes (AG+GG (reference) versus AA) e MTRR genotypes (AA (reference) versus AG+GG). Model 2: Maternal variables as determinants of neonatal tHcy. Independent variables are the same as in model 1. Model 3: Neonatal variables as determinants of neonatal tHcy. Independent variables are the same as in model 1, but from the newborn.

## P075

## EFFECT OF ITALIAN COFFEE INTAKE ON HOMOCYSTEINE, CYSTEINE AND GLUTATHIONE LEVELS IN HEALTHY SUBJECTS

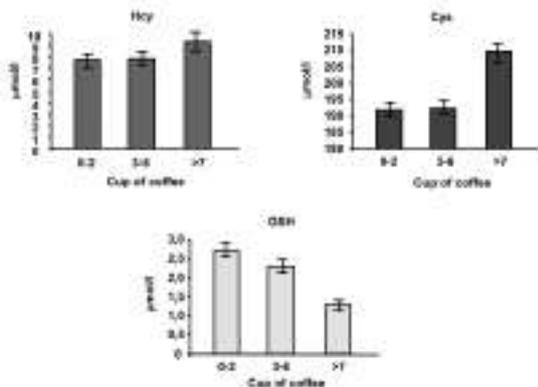
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**Background.** High consumption of coffee is associated with raised concentrations of plasma homocysteine, an independent risk factor of cardiovascular disease. The mechanism by which this popular drink could cause this potentially toxic effect is still unclear. Caffeine, a methyl xanthine can act as vitamin B-6 antagonist, the cofactor of cystathionine-beta-synthase in the transulphuration pathway of homocysteine, so an elevated caffeine intake, could interfere with the irreversible degradation of homocysteine to cysteine. Several studies suggest that caffeine is responsible only for 25-50% of homocysteine increase, therefore, other compounds present in coffee are proposed to explain the correlation between the coffee intake and the homocysteine increase. Among others, chlorogenic acid, a major polyphenol in coffee has been object of major interest; 2 grams this compound increase fasting homocysteine concentration by 4%. Probably, the methylation reactions that occur (happened) during the metabolism of this polyphenol, can induce the increase of homocysteine concentration.

**Aims.** To study the effect of Italian coffee (espresso) on the concentration of homocysteine, cysteine and glutathione in a group of healthy subjects living in Rome.

**Methods.** 1241 healthy volunteers (580 males; mean age 36, interval 18-60) were enrolled for the study. Homocysteine, cysteine and glutathione were measured by HPLC with fluorescence detector according to Araki and Sako. Lifestyle habits were evaluated by an interview questionnaire. The population studied were divided in three different groups on the basis of coffee consumption: 0-2 cup of coffee; 3-6 cup of coffee; and more than 7 cup of coffee a day.



**Results.** People who drink more than 7 cup of coffee a day shows statistically significant increased levels of both homocysteine and cysteine ( $p=0.045$  and  $0.03$  respectively) in respect to the other ones (Figure 1). On the contrary, in the same group a significant reduction of glutathione concentration was found ( $p=0.02$ ).

**Discussion.** Our results shows that the normal consumption of coffee (less than 5/6 cup a day) does not influence the homocysteine concentration nor that of cysteine and glutathione. Coffee interfere with the levels of this compounds only when the intake is over 7 cup a day. The increase of homocysteine, together with cysteine, could not be explained by the vitamin b6 antagonistic effect of caffeine. The significant decrease of glutathione could be explained by its coniugation with chlorogenic acid, as reported by several authors.

**P076**

**GENETIC DETERMINANTS OF HYPERHOMOCYSTEINEMIA IN ATHEROSCLEROSIS**

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Hyperhomocysteinemia (HHcy) is an independent risk factor for the development of atherosclerosis. The mechanisms by which HHcy promotes cardiovascular disease may be due to activation of pro-inflammatory factors, endoplasmic reticulum (ER) stress and oxidative stress. We aimed to study (i) gene mutations that cause HHcy. (ii) Estimation of inflammatory marker like ultrasensitive C-reactive proteins (hs-CRP) and total antioxidant levels (iii) determination of Hcy-dependent gene expression *in vivo*. 25 HHcy patients and 25 healthy controls were taken for this study. Mutation detection in MTHFR, CBS, MS and eNOS gene was by PCR-based restriction enzyme analysis and subsequently expression study was carried out by Reverse Transcriptase PCR and cloning technique. A significant association of HHcy with MTHFR (C677T) and MS (A2756G) genotype was observed ( $p<0.05$ ). There was no association of Hhcy and eNOS genotype. The Hhcy patients, showed no expression of the ER stress gene, GRP78 in lymphocytes. Our Study showed no effect of Hcy on the CD18 gene (pro-inflammatory pathway) expression, but a significant association of tHcy and hs-CRP levels in HHcy grp ( $t=2.28$ ,  $p<0.05$ ). This shows that HHcy induces inflammatory response, which could lead to tissue injury in the pathogenesis of the atherosclerotic process. Our findings show higher mRNA expres-

sion of manganese superoxide (Mn SOD) in HHcy group as compared to the control group. The Total Antioxidant Status (TAS) estimated was found to be significantly lower in the HHcy group as compared to healthy normals ( $t=4.8$ ,  $p<0.01$ ). Taken together these findings strongly suggest that the adverse effects of homocysteine are at least partly mediated by oxidative stress. Our study supports the hypothesis that Hcy evokes adverse vascular effects by promoting oxidative damage to endothelial cells.

**P077**

**INTERACTION OF CHOLINE/BETAINE METABOLISM AND HOMOCYSTEINE PLASMA LEVELS**

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Betaine is an alternate methyl donor for homocysteine remethylation, derived from choline oxidation; betaine homocysteine methyltransferase (BHMT) transfer the methyl group from betaine to homocysteine, resulting in demethylated betaine (dimethylglycine, DMG) and methionine. The influence of betaine supplementation on circulating homocysteine concentration has been studied mainly in clinical settings. Betaine treatment combined with folic acid normalized fasting plasma homocysteine concentrations in patients with severe inborn hyperhomocysteinemia. Little published information is available concerning the effect of betaine on plasma homocysteine concentration in subjects with normal to mild elevated homocysteine levels. Aim of the study was to examine the interaction between homocysteine and choline/betaine metabolism in a group of healthy subjects with normal vitamin status (folic acid, vitamin B6 and B12). We analyzed choline, betaine and DMG in 72 healthy subjects (32F; 40M; median age: 58 years). Determination of choline, betaine and DMG was obtained by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Homocysteine and choline plasma levels were found to be significantly related ( $R=0.33$ ;  $p<0.001$ ), but no significant correlation was observed between homocysteine, betaine and DMG plasma levels. DMG levels were significantly different among males and females ( $1.8\pm0.9$  vs.  $1.4\pm0.5$  uM,  $p=0.01$ ), whereas no significant difference was observed for betaine and choline plasma levels. Choline plasma levels were significantly ( $p<0.001$ ) higher ( $16.7\pm6.8$  µM) in subjects with hyperhomocysteinemia ( $M>15$ ;  $F>13$  µmol/L) respect to those ( $11.3\pm3.1$  µM) with normal values of homocysteine. These preliminary data show the possible interaction between choline and homocysteine levels.

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P078

**THE EFFECT OF VITAMIN SUPPLEMENT ON TOTAL PLASMA HOMOCYSTEINE LEVELS IN ELDERLY SUBJECTS**

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Elevated plasma homocysteine (Hcy) has emerged as a risk factor in a number of diseases, including neural tube defects, vascular disease, Alzheimer's dementia, and osteoporosis. Hcy levels increase with age, and several of Hcy-associated diseases increase in prevalence in elderly individuals. Since the USDA mandated the supplementation of foodstuffs with folic acid, there have been no studies undertaken in the United States to determine treatment effects of different levels of B vitamins and folic acid on serum Hcy levels in an elderly population. We wanted to determine whether low-dose, B vitamin supplementation (folic acid, B12 and B6) or high-dose B vitamins would optimally lower plasma homocysteine levels in an elderly population.

A hundred and thirty-two age and gender matched subjects enrolled in a case-controlled study. Subjects received a low-dose B vitamin supplement for 3 months followed by a high-dose supplement for another 3 months. 109 completed the 6-month, two-phase study during 2002-2005. Fasting plasma was assayed for tHcy, folate, B12 and B6 at baseline, post low-dose B vitamin treatment, and post high-dose B vitamin treatment. Low-dose treatment consisted of 0.4 mg of folic acid, 0.015 mg of B12, and 5 mg of B6 taken once a day for 3 months. High-dose B vitamin treatment was a combination of 2.5 mg of folic acid, 1 mg of B12, and 25 mg of B6 taken once a day for 3 months. Information on use of multivitamins prior to entry into the study was recorded for all subjects. Baseline plasma tHcy levels for the total group of enrollees (mean  $\pm$  SD,  $8.6 \pm 2.6$   $\mu$ mol/L) were substantially lower than reported for similar European or pre-1998 US cohorts (range 10-15  $\mu$ mol/L). A substantial fraction of our subjects used vitamin supplements prior to enrollment. Subjects with prior multivitamin use had slightly lower plasma tHcy at baseline ( $8.2 \pm 2.6$   $\mu$ mol/L) than the non-multivitamin users ( $9.2 \pm 3.0$   $\mu$ mol/L;  $p < 0.07$ ). Low-dose B vitamin treatment had no significant effect on plasma tHcy levels in either multivitamin or non-multivitamin users. However, high-dose B vitamin treatment resulted in further lowering of plasma tHcy in multivitamin users (mean reduction 8.2%;  $p < 0.01$ ) and in the non-multivitamin users (mean reduction 16.5%;  $p < 0.01$ ). Folate, B12 and B6 levels increased 2-4-fold with high-dose vitamin treatment.

Our results indicate that in an elderly population, high-dose B vitamin supplementation is required in order to maximally lower plasma.

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P079

**MTHFR GENOTYPE DISTRIBUTION, VITAMIN B12 AND FOLATE IN HEMODIALYSIS PATIENTS, ORIGINATING FROM CENTRAL- SOUTH ITALY**

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Cardiovascular disease (CVD) is the major cause of death in the general population and in particular in hemodialysis (HD) patients. Hyperhomocysteinemia, a well-recognized cardiovascular risk factor, is frequent in HD subjects. A common polymorphism in the 5,10- methylenetetrahydrofolate (MTHFR) gene is associated with a thermolabile variant of this enzyme which leads to elevated Hcy levels. Purpose of the study was to investigate whether MTHFR polymorphism, vitamin B12 and folate are capable of influencing Hcy concentrations also in HD patients. Effects of MTHFR genotype, vitamin B12, and folate on plasma Hcy levels were examined in 132 HD patients, 72 male and 60 female, not under current treatment with folates and/or Vit B12.

MTHFR polymorphism was based on PCR amplification. Vit B12 and folate levels were detected on Modular E (Roche). Hcy levels were assayed using HPLC with fluorometric detection.

The genotype frequency were 0.38, 0.43 and 0.19 for CC, CT and TT carriers respectively. The corresponding mean Hcy levels in microM/L were 26,96 ( $\pm 13,28$  SD), 55,19 ( $\pm 36,71$  SD), 100,13 ( $\pm 60,72$  SD). On ANOVA these differences were statistically significant ( $p < 0.0045$ ). Both folates and Vit B12 were highly correlated with Hcy levels ( $p < 0.00058$  and  $< 0.006$ ). On multiple regression analysis only the MTHFR genotype and folates were associated with Hcy levels.

These preliminary data seem to indicate that: 1) Hcy levels increase almost five-tenfold in HD subjects when compared to a healthy population; 2) the MTHFR polymorphism is highly influencing Hcy levels.

P080

**HOMOCYSTEINE AND RELATED B VITAMIN CONCENTRATIONS IN BRITISH CHILDREN AND ADOLESCENTS**

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*Background.* Age and sex specific reference data for homocysteine and its B vitamin determinants have not been widely investigated in children.

*Aims.* To investigate the effect of age, sex and relevant B vitamin status on plasma total homocysteine concentrations (tHcy) in children and adolescents.

*Methods.* Data from the National Dietary and Nutritional Survey (NDNS) of young people aged 4-18 years (1997) were analysed to provide a nationally representative sample within the British population.

*Results.* As tHcy increased with age, there was a corresponding decrease in the status of all related B vitamins. Additionally (not shown), no difference in tHcy between males and females in the two younger age groups was found, but among 15-18 year olds, males had a significantly higher tHcy ( $p = 0.026$ ) despite having significantly higher status of red cell folate and riboflavin (in all age groups) and

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of PLP (15-18 year olds only).

**Conclusions.** It is not generally appreciated that the expected age-related increase in tHcy reflects decreases in all four B vitamin determinants from 4-18 years. These findings suggest a need to establish age specific reference ranges for homocysteine and related B vitamin concentrations within a pediatric setting.

**Table 1.**

	4-10y n=562	11-14y n=374	15-18y n=344	p
Age (y)	8.04(6.2-9.5) <sup>a</sup>	12.8(11.8-13.9) <sup>b</sup>	16.9(15.9-17.9) <sup>c</sup>	0.000
tHcy(μmol/l)	5.6(4.4-6.7) <sup>a</sup>	6.3(5.2-7.7) <sup>b</sup>	7.9(6.3-10.3) <sup>c</sup>	0.000
Red cell folate (nmol/L)	624(522-778) <sup>a</sup>	540(435-689) <sup>b</sup>	490(395-613) <sup>c</sup>	0.000
Serum Folate (nmol/L)	24.3(18.6-29.5) <sup>a</sup>	19.3(14.6-24.9) <sup>b</sup>	15.6(11.7-21.5) <sup>c</sup>	0.000
Serum B12 (pmol/L)	463(343-577) <sup>a</sup>	338(254-447) <sup>b</sup>	261(195-349) <sup>c</sup>	0.000
PLP (nmol/L) 1	59.1(44.6-75.3) <sup>a</sup>	55.7(42.3-73.7) <sup>ab</sup>	52.85(39.4-74.6) <sup>b</sup>	0.026
EGRac2	1.38(1.31-1.47) <sup>a</sup>	1.45(1.35-1.58) <sup>b</sup>	1.47(1.37-1.61) <sup>b</sup>	0.000

Values are median (IQR). a, b, c Unlike superscripts indicate significant differences between age groups (ANOVA, with LSD test). Skewed variables were log transformed. 1Pyridoxal-5-Phosphate; vitamin B6 status. 2Erythrocyte glutathione activation coefficient; a higher value indicates lower riboflavin status.

## P081

### RED BLOOD CELL FOLATE, HOMOCYSTEINE, COBALAMIN AND METHYL MALONIC ACID THROUGH THE FOLIC ACID FORTIFICATION YEARS

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Folic acid fortification (FAF) became mandatory in Canada in November 1998. The long-term effects of FAF on intracellular folate (RBC folate) homocysteine (tHcy) cobalamin (Cbl) and methylmalonic acid (MMA) were examined in a cohort of older adults. The cohort baseline started in 1997 and participants were tested twice subsequently (until December 2004). THcy and MMA were determined by mass spectrometry gas-chromatography. Scatter plots demonstrated trends in RBC folate, tHcy, Cbl and MMA over time. SAS was used for statistical analysis. Piecewise linear and polynomial regression were used to allow the slopes to vary over time, and when modeling the relationships between RBC folate and tHcy. A mixed effects model was used to account for the within subject correlation, induced by the repeated measures design. RBC folate levels increased steadily through the five years and have not reached a plateau (neither in participants taking multivitamins or in the ones without voluntary supplementation) (average RBC folate in 2004 was 3.5 times higher than in 1998). By 2002, 91% of participants already had RBC folate levels above the normal range. Levels of tHcy declined initially to a nadir between 1999 and 2000, but increased significantly to levels similar to the pre-fortification period by 2002 ( $p < 0.005$ ) and continue to increase. Cbl levels increased through the years, as more participants were on multivitamin supplements. MMA levels were higher in 2004 than 1998 and were inversely correlated to Cbl levels with a 12% decrease in MMA for each 1% of increase in Cbl.

**Conclusions.** 1. In this population FA accumulates intracellularly. 2. THcy levels returned to pre-fortification levels in spite of continuing increase in RBC folate, therefore, reduction of tHcy levels with FA is limited and perhaps reaches a threshold. 3. MMA levels increased with FAF.

## P082

### EFFECT OF A DIETARY INTERVENTION ON METHIONINE/HOMOCYSTEINE KINETICS

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**Background.** It was reported that soy-based foods could reduce homocysteine concentrations because they are rich in folates and phytoestrogens and because soy proteins are poor in methionine.

**Aims.** To evaluate the effects of a dietary intervention based on the substitution of animal-derived proteins with soy-derived proteins on nutritional status and on the kinetics of homocysteine and methionine.

**Methods.** Eight subjects underwent two tracer studies of methionine/homocysteine kinetics after two 3-week dietary periods in randomized order: control diet and an isocaloric diet in which the animal proteins were substituted by soy derived foods proteins. To measure the whole-body rates of methionine flux and methionine cycling through homocysteine (remethylation pathway), each study begun with the prime infusion of the tracers [methyl-2H3, 1-13C]methionine and [2H3]leucine and was followed by a continuous infusion of [methyl-2H3, 1-13C]methionine and [2H3]leucine for 3.5 hours. Biochemical parameters of nutritional status and body composition analysis (DEXA) were performed prior to and after each dietary intervention. **Results.** The dietary intervention decreased by 16% both homocysteine and methionine concentrations without changing the rate of methionine remethylation, but increasing by 43% the efficiency at which homocysteine is recycled. Proteolytic rate and fat-free and fat mass were unchanged. It is noteworthy that this results was obtained with a modest increment in the concentration of folic acid and despite a minor (non significant) decrement in the concentration of B12 vitamin, and no other changes in measured parameters of nutritional status.

**Conclusions.** it is feasible to reduce homocysteine concentrations with a soy-based dietary intervention by increasing the efficiency of methionine recycling. This evidence supports the need for a dietary trial study in subjects with mild elevations in homocysteine concentrations.

**Table 1.**

dietary period	Concentrations (μmol/L)		Methionine kinetics		Protein metabolism
	homocysteine	methionine	methyl flux	efficiency of meth remeth	breakdown
soy	10,24±1,517	23,44±1,563	21,6±0,7	0,387±0,034	138,4±8,7
control	8,653±1,6	19,95±1,084	23,9±1,0	0,551±0,06	135,4±12,5
% change	-16%±6%	-14%±5%	10%±3%	43%±10%	-2%±5%
p	0.031	0.038	0.016	0.008	0.715
dietary period	folic acid ng/ml	vit B12 pg/ml	vit B6 ng/ml	triglycerides mg/dl	cholesterol mg/dl
soy	5,352±0,235	542,2±153,2	74,7±11,1	96±14,7	163,0±11,2
control	6,567±0,564	432,4±110,8	72,9±8,3	88,2±6,719	155,0±12,5
% change	22%±7%	-16%±7%	2%±7%	0%±16%	-5%±4%
p	0.025	0.082	0.076	0.663	0.236

## P82a

## THIOLS, ENERGETIC AND REDOX STATUS IN HEAVY SMOKERS

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**Background.** Oxidative stress, induced by smoking, enhances the progression of cardiovascular diseases. Thiols, energetic and redox status maintain the efficiency of the intracellular antioxidant system which counteracts oxidative stress.

**Aims.** To investigate the relationship between smoking and the above mentioned metabolic aspects.

**Methods.** Whole blood and plasma thiols, both total and reduced, [glutathione (GSH), cysteine (Cys), cysteinylglycine (CysGly), homocysteine (Hcy)], erythrocyte redox (NADH, NAD<sup>+</sup>; NADPH, NADP<sup>+</sup>) and energetic (ATP, ADP and AMP) status, serum Reactive Oxygen Species (ROS) levels and Total Antioxidant Capacity (TAC) were compared in 54 healthy subjects: 33 heavy smokers (HS, 55.4±5.4 years) and 21 non-smokers as controls (C, 47.3±18.4 years). ROS and TAC levels were measured by the relevant spectrophotometric assays; all the other analytes were determined by HPLC. Statistical analysis was performed by univariate and multivariate analysis.

**Results** are reported as mean ± SD:

Subjects	HS	C
plasma tHcy (microM)	15.0±9.4*	9.9±3.1
plasma red CysGly (microM)	6.2±4.1°	3.9±1.4
ery-ATP (microM)	1765.9±323.3#	1998.3±239.2
ery-NADH (microM)	9.8±6.5§	13.9±6.1

\**p* = 0.03 vs C, °*p* = 0.02 vs C, #*p* = 0.014 vs C, §*p* = 0.04 vs C

Plasma total Hcy and reduced CysGly levels were significantly higher in HS than in C. No difference was found in GSH and Cys levels. Both energetic and redox status were impaired in smokers compared to controls, being ery-ATP and ery-NADH levels significantly lower in HS than in C. Circulating parameters of oxidative status (ROS and TAC) showed no difference between the two groups.

**Conclusions.** Heavy smokers were characterized by alterations in thiol levels and in both energetic and redox status, already evident at intracellular but not yet at peripheral level. Our findings, therefore, confirm that smoking is related to oxidative stress and may be considered an additional risk factor for cardiovascular diseases.

## P083

## INTRACELLULAR S-ADENOSYLHOMOCYSTEINE ACCUMULATION TRIGGERS GLOBAL DNA HYPOMETHYLATION IN HUVEC

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**Background.** Increasing attention has been focusing on an indirect mechanism for homocysteine (Hcy) toxicity, secondary to S-adenosylhomocysteine (AdoHcy) -mediated inhibition of the DNA methyltransferases and affecting DNA methylation patterns. Methylation is a major epigenetic feature of genomic DNA, leading to alterations in gene expression. *In vivo* evidence has been gained suggesting the AdoHcy ability to affect DNA methylation status. However, the effect of intracellular AdoHcy accumulation on DNA methylation patterns has not yet been fully substantiated by experimental evidence.

**Aims.** The present study was designed to evaluate, in human umbilical vein endothelium cells (HUVEC), the effect of intracellular AdoHcy accumulation on global genomic DNA methylation status.

**Methods.** Experimental intracellular accumulation of AdoHcy was induced by adenosine-2,3-dialdehyde (ADA), an inhibitor of S-adenosylhomocysteine hydrolase. Increasing concentrations of inhibitor were tested and un-supplemented medium incubations were used as controls. Cytosolic and nuclear fractions were obtained from trypsinized cells, after 72 h of incubation. Total homocysteine (tHcy) concentration was quantified (culture medium and cytosolic fractions) by HPLC. S-adenosylmethionine (AdoMet) and AdoHcy concentrations were measured (cytosolic fractions) by stable-isotope dilution LC-tandem mass spectrometry method. Genomic DNA was obtained from the nuclear fraction and global DNA methylation status was evaluated by the cytosine extension assay.

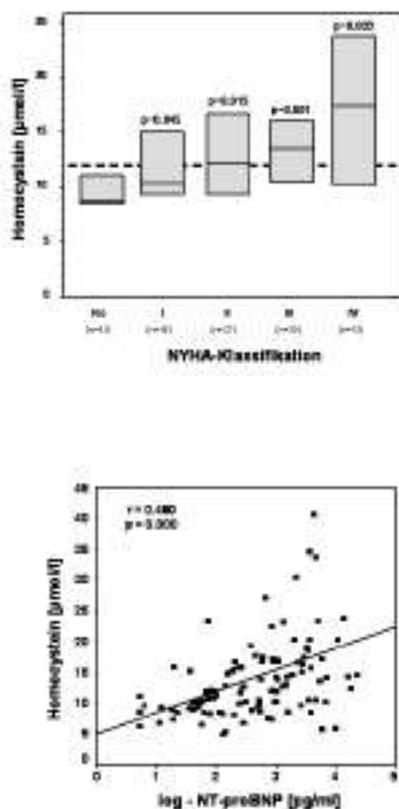
**Results.** The results showed that supplementation of the culture medium with ADA had no cytotoxic effect and increased the intracellular AdoHcy concentration in a dose-dependent manner. Correlation analysis showed the presence of (1) a negative correlation between intracellular tHcy and intracellular AdoHcy concentrations (*r* = -0.92; *p* < 0.00001); (2) a positive correlation between intracellular AdoHcy concentration and [<sup>3</sup>H]-dCTP incorporation (*r* = 0.84, *p* < 0.0001); and (3) a negative correlation between intracellular AdoMet/AdoHcy ratio and [<sup>3</sup>H]-dCTP incorporation (*r* = -0.83; *p* < 0.0001). In addition, intracellular AdoMet concentration remained constant.

**Conclusions.** These findings strongly point to the importance of intracellular AdoHcy as a biomarker of genomic DNA methylation status.

P084

**RELATION OF PLASMA HOMOCYSTEINE TO THE SEVERITY OF CHRONIC HEART FAILURE**Herrmann M,<sup>1</sup> Kindermann I,<sup>2</sup> Müller S,<sup>1</sup> Georg T,<sup>3</sup> Kindermann M,<sup>2</sup> Böhm M,<sup>2</sup> Herrmann W<sup>1</sup><sup>1</sup>Department of Clinical Chemistry and Laboratory Medicine, University Hospital of Saarland, Homburg/Saar, Germany; <sup>2</sup>Department of Internal Medicine III, University Hospital of Saarland, Homburg / Saar, Germany; <sup>3</sup>Department of Medical Biometry, Epidemiology and Medical Informatics, University Hospital of Saarland, Homburg/Saar, Germany

Hyperhomocysteinemia (HHcy) has been suggested as a cardiovascular risk factor. It can be speculated that elevated plasma homocysteine (HCY) is also related to the severity of CHF. This study aimed to analyze the relation between HCY and the severity of CHF using clinical, echocardiographic and laboratory parameters of CHF. We investigated 95 CHF patients (54±13 years) and 18 healthy controls (44±10 years). All subjects underwent echocardiography, venous blood sampling and a 6-minute-walk-test (6-MWT). In 82 patients cardiac catheterization was performed. Additionally, 37 patients underwent spirometry to determine maximum oxygen uptake (VO<sub>2</sub>max).



Serum HCY, N-terminal pro-brain natriuretic peptide (NT-proBNP) and creatinine were studied. HCY ( $p=0.002$ ), NT-proBNP ( $p<0.001$ ), creatinine ( $p=0.001$ ) and left ventricular enddiastolic diameter ([LVDD],  $p<0.001$ ) increased with increasing NYHA-classes. Contrary, VO<sub>2</sub>max ( $p=0.026$ ), 6-MWT ( $p<0.001$ ), and ejection fraction ([EF],  $p<0.001$ ) decreased with increasing NYHA-classes. HCY was signifi-

cantly correlated with VO<sub>2</sub>max ( $r=-0.528$ ,  $p=0.001$ ), 6-MWT ( $r=-0.285$ ,  $p=0.007$ ), NT-proBNP ( $r=0.434$ ,  $p<0.001$ ), LVDD ( $r=0.326$ ,  $p<0.001$ ) and EF ( $r=-0.202$ ,  $p<0.050$ ). After correction for age and creatinine, NT-proBNP ( $p=0.002$ ) and LVDD ( $p=0.041$ ) were significantly associated with HCY. The present study demonstrates that HCY is related to clinical, echocardiographic and laboratory parameters of CHF suggesting a relation between HCY and the severity of CHF.

P085

**HYPERHOMOCYSTEINEMIA (HHcy) AS A RISK FACTOR OF BONE FRACTURE IN ELDERLY WOMEN**

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Chronic exposition to hyperhomocysteinemia (HHcy) may induce degenerative alterations in connective tissue of bones; hence HHcy it has been suggested as a possible risk factor for bone fractures in the elderly, where HHcy is known to have a high prevalence. Aim of our study is to obtain more insight in this finding.

**Materials and Methods.** In 233 elderly women (age 61-88 yr), nutritional status (BMI index, serum albumin), total plasma fasting homocysteine (Hcy), biochemical parameters known to influence Hcy levels (RBC folate, serum vitamin B12, creatinine, TSH), number of diseases (CIRS), physical performance status (ADL and IADL score), clinical history (number of falls and fractures, drugs), parameters of bone mass and body composition (bone density and body lean/fat mass percentage by DXA) and cognitive screening (MMSE score) were assessed.

**Results.** The subjects in the highest Hcy tertile ( $H_e > 16$  micromol/L) resulted significantly older ( $84 \pm 6$  yr), more disabled (reduced ability to perform movements and tasks) (mean score ADL 2/6; IADL 1/8) with respect to other groups; moreover, they showed a poorer nutritional status (significantly lower serum albumin, folate and vitamin B12 levels) and higher cognitive derangement (>60% had MMSE score < 21); differently from the other 2 groups, in the group with HHcy, Hcy levels resulted significantly correlated to a higher number of falls in the last 3 years and to an higher percentage of bone fractures, including also spine fractures. Within the different considered parameters, besides MMSE and ADL-IADL score and low mean body mass, HHcy resulted significantly associated to a significant bone loss (spine and femur T score < -1.5) (OR= 2.56, CI 1.67-5.67 95% CI,  $p<.01$ ).

**Conclusions.** Hyperhomocysteinemia in the elderly is associated to a higher prevalence of falls and fractures; these occurs when elderly have a reduced physical and cognitive performances. According to our study, HHcy should be taken in account as an aspecific marker of disability in the elderly.

P086

**ELEVATED PLASMA HOMOCYSTEINE LEVELS ACCOMPANIED BY OSTEOPOROTIC HIP FRACTURE IN ELDERLY PATIENTS**Cho Karl JW, Norling A, Berg A, Cederholm T, Lökk J  
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**Background.** Genetic hyperhomocystinuria is a biological abnormality associated with skeleton abnormalities and osteoporosis. Plasma homocysteine increases progressively with age often due to cobalamine and/or folate deficiency mainly related to malabsorption caused by atrophic gastritis. Age-related decline in glomerular filtration rate also cause elevation in plasma homocysteine. Hyperhomocysteinemia covaries in a number of diseases in late life, including senile osteoporosis. Mildly elevated homocysteine levels might be related to age-related osteoporotic fractures and also appears to be an independent risk factor for osteoporotic fractures in elderly. Hyperhomocysteinemia can influence not only bone mineral components, but also soft components in bone tissue. Several studies recently suggested that high plasma homocysteine may weaken bone tissue by interfering with collagen cross-linking, thereby increasing the risk for osteoporotic fractures.

**Aims.** The aim of the study was to assess plasma homocysteine levels in elderly patients (above 65 years) admitted and operated on low-energy fractures with relation to osteoporosis and to correlate homocysteine levels with cobalamine and folate, nutrition state, bone mineral density and renal function.

**Table. Investigated parameters, mean values  $\pm$  SD and numbers**

Parameters		Mean value and numbers
	Homocysteine (mmol/L)	17 $\pm$ 54; 11/30 >20 mmol/L
	Cobalamine (mmol/L)	287 $\pm$ 86; 18/30 >650 mmol/L
<b>Nutrition state</b>	BMI	22 $\pm$ 3.7
	Albumin (g/L)	32 $\pm$ 5.9
	IGF-1 ( $\mu$ g/L)	63 $\pm$ 12
<b>Renal function</b>	GFR	62.6 $\pm$ 26
	S-Creatinine (mmol/L)	72 $\pm$ 13
<b>Bone mineral density</b>	Total mass (kg)	59 $\pm$ 13
	Fat mass (kg)	19 $\pm$ 6.3
	Lean mass (kg)	38 $\pm$ 10
	T score > -1	4/30
	T-score -1 - -2.5	4/30
	T-score < -2.5	22/30

**Methods.** Patients with low-energy hip fractures were consecutively recruited from a gero-orthopedic ward after informed consent. Nutrition state of patients were determined by body mass index (BMI), plasma albumin and Insulin like growth factor 1 (IGF-1). Blood samples were collected while patients were in non-fasting status. Bone mineral components, fat and lean body mass were determined by dual-energy X-ray absorptiometry Lunar (DXAL) system at whole body and unfactured hip. Renal function was calculated by glomerular filtration rate (GFR) by Cockcroft-Gaults formel: 1.22(man) or 1.04(woman) x 140 - age x body weight divided by plasma creatinine.

**Results.** The study comprised 30 patients; 25 women (83%) and 5 men (17%), mean age 76 years and 2 months (Table). No correlations between homocysteine and cobalamine or folate were found. There was a slightly negative correlation between homocysteine and BMI as well as negative correlation between homocysteine and body mineral density and slightly positive between plasma homocysteine and GFR.

**Conclusions.** Our elderly patients with osteoporotic hip fracture had moderately elevated plasma homocysteine levels without evidence of cobalamine or folate deficiency. Plasma homocysteine elevation was related to malnutrition and to decline in GFR. Elevated plasma homocysteine also correlated to bone mineral density and fat body components. We suggest that plasma homocysteine might be a predictive risk factor of age-related osteoporotic hip fracture. Elevated plasma homocysteine levels in combination with malnutrition call for investigation of osteoporosis.

P087

**EFFECT OF ROSIGLITAZONE ON HOMOCYSTEINE METABOLISM IN ZDF (TYPE 2) DIABETIC RATS**Wijekoon EP, Hall B, Ratnam S, Brosnan ME, Brosnan JT  
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Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. We have previously shown that both insulin resistance and frank type 2 diabetes have marked effects on homocysteine (Hcy) metabolism. The present study was conducted to determine the effects of the thiazolidinedione, rosiglitazone, on these changes. Male ZDF/Gmi fa/fa and ZDF/Gmi fa/+ rats aged 6 weeks were fed either chow (control) or chow mixed with rosiglitazone (RSG) to give 10  $\mu$ mole/kg body weight/day and were studied before the start (6 wks old) or after 6 weeks (12 wks old) of treatment. At 6 weeks of age, the ZDF fa/fa rats needed 5 fold more plasma insulin to maintain plasma glucose comparable to the ZDF fa/+ rats. At 12 weeks of age, the ZDF fa/fa (control) rats were insulin-resistant and diabetic, while the ZDF fa/fa (RSG) rats maintained a normal glucose level with only 37% of the insulin observed at 6 weeks. The plasma total Hcy which showed a 44% reduction in the ZDF fa/fa (control) rats returned towards normal after RSG treatment. The increased activities of methionine adenosyltransferase and cystathionine gamma-lyase in the ZDF fa/fa (control) rats were corrected by the RSG treatment. Interestingly, the increased activity of betaine: homocysteine methyltransferase (BHMT) observed in the ZDF fa/fa rats was not corrected; in fact, it was highest in the ZDF fa/fa (RSG) group. Hepatic S-adenosyl methionine (SAM) was significantly higher in the ZDF fa/fa (control) rats compared to the ZDF fa/+ (control) rats, but the SAM level in RSG treated rats did not differ from either set of control rats. Since RSG acts by increasing insulin sensitivity, this study confirms that insulin plays a major role in the regulation of hepatic Hcy metabolism. However, the regulation of BHMT expression appears to be complex.

Supported by CDA and CIHR.

P088

**HOMOCYSTEINE PLASMA LEVELS AND MTHFR GENOTYPE IN PATIENTS WITH RETINAL VEIN OCCLUSION**

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Many recent scientific reports have suggested that hyperhomocysteinemia, a recognized independent risk factor for vascular disease, may be associated with an increased risk of retinal vein occlusion (RVO). We studied 69 patients (mean age 64.1 $\pm$ 14.6 y; 43 males: 58.2 $\pm$ 14.4 y; 53 females: 68.9 $\pm$ 13.0 y) with RVO, dosing plasma levels of homocys-

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teine (tHcy), fasting and after loading with methionine (FPIA-Abbot), cyanocobalamine and folic acid levels (CMIA-Abbot) and testing methylenetetrahydrofolate reductase (MTHFR) C677T mutation (Light Cycler-Roche). We used 50 healthy subject as a control group. Fasting levels of tHcy were significantly higher in patients than in controls: mean value 14.3 vs 10.6 nmol/mL (cut off: M 16 nmol/mL, F 13 nmol/mL);  $p=0,02$ ; Post load levels were also significantly higher: mean value 42.1 vs 30.4 nmol/mL (cut-off: M 41 nmol/mL, F 38 nmol/mL;  $p<0.001$ ); tHcy increase after methionine load was significantly higher: mean  $\Delta$ tHcy 27.5 vs 19.8 nmol/mL (cut-off 25 nmol/mL,  $p=0.007$ ). The prevalence of high levels for fasting tHcy, postload tHcy and  $\Delta$ tHcy was respectively 46.9% (vs 5.2%,  $p<0,0001$ ); 55.2% (vs 12.1%,  $p<0.0001$ ) and 54.2% (vs 15.5%,  $p<0.0001$ ). Furthermore 3/58 (5%) patients had low vitamin B12 levels, and 11/58 (19%) had low levels of folic acid, 6 of these having high fasting tHcy (55%). Heterozygous and homozygous MTHFR mutation were respectively 29/63 (46%) and 18/63 (29%), being in the literature in healthy subjects 40% heterozygous and 12% homozygous. Our data suggest that hyperhomocysteinemia is a risk factor for RVO, that homozygous mutation and high levels of tHcy are more frequently associated with RVO: it has to be confirmed if the mutation is per se a risk factor for RVO.

**Conclusions.** screening for tHcy plasma levels should be recommended in patients with recent RVO, and vitamin supplementation, that is known to reduce tHcy levels, could be usefully associated with antithrombotic therapy.

### P089

#### HYPERHOMOCYSTEINEMIA IN A BOY WITH JUVENILE OSTEOPOROSIS

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**Background.** Juvenile idiopathic osteoporosis (JIO) is a heterogeneous multifactorial disease with clinical manifestation between 5th and 17th year of life (abnormal gait, pains in the feet, spine, hips; progressive deformities of spine and extremities). Toxic effect of hyperhomocysteinemia (HHC) was proved outside endothelial cells and coagulation proteins also in other tissues of mesenchymal origine. The mechanism has been explained by its very active -SH groups interfering with crosslinking of collagen or elastin mediated by aldehydic groups. In our previous study a higher levels of tHcy were observed among children suffering from bone dysplasias ( $6,6\pm 3,6$   $\mu$ mol versus  $5,4\pm 1,3$   $\mu$ mol/L). That enabled us to present a boy with JIO and severe HHC.

**Case Report.** 16 yrs old boy was referred to osteological examination because of repeated fractures of upper extremities; from 11 to 16 years he sustained 7 fractures. Proband was born after physiological pregnancy; at the age of 16 years he has asthenic habitus, height 186 cm/weight 64 kg; A mild sinistroconvex scoliosis of thoracic spine, accentuation of thoracic kyphosis and lumbar lordosis; mild thoracic dextro-convex and sinistro-convex scoliosis, lumbar dextro-scoliosis. Thoracic kyphosis due to wedge shaped vertebral bodies (T5, T7 and T8). Abnormal pattern of bone structure of thoracic and lumbar vertebral bodies (vertical striation), that framed up. X-ray of left hand "marfanoid" shape, abnormal pattern of bone texture of distal radius, ulna and carpal bones. Mineral, protein, lipid markers within normal limits. Osteocalcine 46,9  $\mu$ g/L; bone ALP 42.9 U/L; pyrridinoline 91,8 and deoxypyrridinoline 10,4  $\mu$ mol/mmol creat.; parathormon 26 pg/mL, vitamin D3 63,8  $\mu$ mol/L, FT4 16,8 pmol/L; increased tHcy 43,8  $\mu$ mol/L and 57,8  $\mu$ mol/L. S-

Met-72  $\mu$ mol/L; L-Met loading test: 77,4  $\mu$ mol/L tHcy 6 hrs after load; folate: 8,3 nmol/L; B 12: 154 pmol/L; B 6: 16,9  $\mu$ g/L; MTHFR 677-TT homozygous status.

**Summary.** According to radioclinical and biochemical examination of bone turnover we concluded diagnosis as JIO and the treatment with Cholecalciferolum and Calcium was introduced accompanied with 5 mg/d folate and 300  $\mu$ g/w of B12 that normalised promptly HHC as well as markers of bone metabolism.

### P090

#### RELATIONSHIP BETWEEN HYPERHOMOCYSTEINEMIA (HHCY) AND BONE LOSS DEGREE IN ELDERLY PATIENTS WITH AND WITHOUT OSTEOPOROSIS

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**Background.** Recently hyperhomocysteinemia (HHcy) has been indicated a possible risk factor for bone fractures in the elderly. Methionine load test is used to assess Hcy metabolism. The aim of the study was to describe the relationship between levels of Hcy before and after methionine load in patients with and without bone loss.

**Methods.** Ninety-nine women (mean age  $71.7\pm 13.5$ ) were submitted to total-body, dual-X-ray absorptiometry (Lunar DXA) to evaluate body composition and bone density. Six blood samples were taken for each patient to measure plasma Hcy (basal and after methionine load-100 mg/kg body weight). We divided patients in three groups, according to the T-score obtained at DXA:  $>-1.5$  (Normal, N=59),  $1.5-2.5$  (Osteopenia, N=21),  $<-2,5$  (Osteoporosis, N=19).

**Results.** The results are described in the table. Patients with osteoporosis show higher values of Hcy, both before and after methionine load, while plasma levels of vitamin B12 and folates are inversely related to bone mineral density.

**Conclusions.** Together with folates and vitamin B12, both plasma levels of Hcy before and after methionine load are associated to the degree of bone loss in old women. Preventive measures to improve Hcy metabolism could positively influence bone mineral density and possibly reduce the risk of fracture in the elderly.

Table 1.

	Normal (N=59)	Osteopenia (N=21)	Osteoporosis (N=19)	P for trend
Basal HCY ( $\mu$ mol/L)	11.3 $\pm$ 3.4	15.5 $\pm$ 7.1	26.2 $\pm$ 8.3	0.008
2 hrs ( $\mu$ mol/L)	23.4 $\pm$ 3.4	27.9 $\pm$ 10.1	40.4 $\pm$ 17.7	0.020
4 hrs ( $\mu$ mol/L)	23.6 $\pm$ 7.5	31.9 $\pm$ 9.9	43.4 $\pm$ 22.9	0.037
6 hrs ( $\mu$ mol/L)	24.7 $\pm$ 4.2	37.8 $\pm$ 12.2	52.1 $\pm$ 26.1	0.047
8 hrs ( $\mu$ mol/L)	39.2 $\pm$ 12.5	39.3 $\pm$ 8.5	61.0 $\pm$ 24.2	0.045
24 hours ( $\mu$ mol/L)	27.2 $\pm$ 11.8	31.4 $\pm$ 20.4	54.0 $\pm$ 20.5	0.035
Vitamin B12 (pg/mL)	683.3 $\pm$ 177.4	516.5 $\pm$ 163.5	228.1 $\pm$ 165.1	0.021
Folic acid (ng/ml)	10.2 $\pm$ 2.9	8.4 $\pm$ 2.1	3.9 $\pm$ 1.9	0.013

P091

**HOMOCYSTEINE AND GLUCOSE HOMEOSTASIS IN OBESE CHILDREN**

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**Background.** Fat-rich diet associates to hyperhomocysteinemia in healthy adults, probably owing to its vitamin low content. Fat-rich diet associates to obesity in children, which is also related with cardiovascular risk factors, such as hyperinsulinism with insulin resistance, hyperlipidemia and arterial hypertension. Nevertheless, there are few data in the literature about a possible relationship between homocysteine and anthropometric data or glucose homeostasis markers in obese children.

**Aims.** To evaluate the possible relationship between homocysteine, anthropometric data and glucose homeostasis markers (insulin and C peptide) in obese children and adolescents.

**Patients.** Seventy-eight obese patients (6-18 years old), 27 of them with acanthosis nigricans. Homocysteine results were compared with those of healthy age-matched controls.

**Methods.** Plasma total homocysteine (tHcy) concentrations were measured by HPLC with fluorescence detection of the SBDF derivatives. Insulin and C peptide were analysed by chemiimmunoluminescence. Insulin resistance was evaluated by the Homeostatic Model Assessment (HOMA). **Results.** Obese children without acanthosis older than 10 years of age showed tHcy levels significantly higher ( $p < 0.0001$ ) than age-matched controls ( $9.0 \pm 2.4$  vs  $6.9 \pm 1.4$   $\mu\text{mol/L}$ ). Children with acanthosis showed an insulin resistance, obesity score (Quetelet index) and hyperinsulinism significantly higher ( $p = 0.01$ ) than obese children without acanthosis. However, tHcy levels were significantly lower ( $p = 0.018$ ) in obese children with acanthosis compared with those without it ( $6.3 \pm 1.6$  vs  $7.4 \pm 2.6$   $\mu\text{mol/L}$ ). A positive correlation was observed between tHcy and C peptide values or Quetelet index in obese children without acanthosis ( $p < 0.002$ ), while this correlation was only observed between tHcy and C peptide ( $p = 0.034$ ) in patients with acanthosis. No other biochemical markers of metabolic syndrome were found.

**Conclusions.** Obese children without acanthosis had a tendency to moderate hyperhomocysteinemia, which is associated with obesity score and C peptide values. The marked hyperinsulinism observed in obese children with acanthosis nigricans seems to decrease tHcy levels.

P092

**STAGE 1 OF THE CARDIOVIT STUDY: CARDIOVIT, ATHEROSCLEROTIC VASCULAR DISEASE AND HYPERHOMOCYSTEINEMIA: AN EPIDEMIOLOGICAL STUDY IN INDIANS, ADDITIONALLY EVALUATING THE EFFECT OF ORAL VITAMIN SUPPLEMENTATION**Rajadhyaksha VD,<sup>1</sup> Desai CD,<sup>1</sup> Shenoy KT,<sup>2</sup> Lena KB,<sup>2</sup> Shaikh AA,<sup>1</sup> Sali N,<sup>2</sup> Shenoy ST<sup>2</sup><sup>1</sup>Medical Research Division, Pfizer Ltd, Mumbai; <sup>2</sup>Medical College, Trivandrum, India

**Background.** Hyperhomocyst(e)inemia (hypertHcy) is emerging as a novel independent risk factor for atherosclerotic vascular disease (AVD) in Indians. An increasing evidence of its association with CHD in subjects of Indian origin is seen, possibly because of inadequate folic acid and vitamin B12 intake. However there is a lack of epidemiological data on its prevalence in Indians.

**Aims.** CARDIOVIT study was divided into two stages. The primary objective of Stage 1 was to estimate the prevalence of hypertHcy in the selected population. The secondary objectives were to assess the association between hypertHcy and AVD, including coronary, cerebral, and peripheral vascular disease, and other conventional and non-conventional risk factors of AVD.

**Methods.** Non-interventional cross-sectional epidemiological study, which involved a one-time screening of randomly selected subjects (age from 25-64 years) in rural and urban areas of the southern Indian state of Kerala. Screening involved medical history, examination, administration of epidemiological and food questionnaire and assessment of blood levels of various parameters including plasma homocyst(e)ine (tHcy).

**Results.** 1714 individuals were evaluated. tHcy > 12 mmol/L was reported in 872 (50.9%) individuals. Prevalence was higher in males (505/802; 62.96%) compared to females (367/910; 40.32%). Positive association of hypertHcy with age, mean corpuscular volume, smoking, hyperlipidemia, alcohol intake, tobacco use, and negative association with diabetes mellitus were seen. Results of the regression analysis showed gender to have a significant ( $p < 0.001$ ) association with hypertHcy. Age had a marginally significant ( $p = 0.067$ ) positive association, whereas diabetes mellitus had a significant ( $p = 0.002$ ) negative association. tHcy was higher in smokers vs non smokers (15.4 vs 12.67  $\mu\text{mol/L}$ ) and hyperlipidemics vs normolipidemics (14.03 vs 13.35  $\mu\text{mol/L}$ ).

**Conclusions** This largest epidemiological study in India till date showed that hypertHcy is widely prevalent in India and is associated with some of the other traditional risk factors of AVD. Steps need to be taken to manage this emerging risk factor.

P093

**INTERRELATIONSHIPS BETWEEN B VITAMIN STATUS, PLASMA TOTAL HOMOCYSTEINE (tHcy), AND MARKERS OF BONE HEALTH IN POSTMENOPAUSAL WOMEN**Walsh PM,<sup>1</sup> Strain JJ,<sup>1</sup> McNulty H,<sup>1</sup> O'Connor JM,<sup>1</sup> Ueland PM,<sup>2</sup> Scott JM,<sup>2</sup> Bradbury T<sup>2</sup><sup>1</sup>Northern Ireland Centre for Diet and Health, University of Ulster, Coleraine; <sup>2</sup>Department of Biochemistry, Trinity College Dublin, Ireland; <sup>3</sup>Locus for Homocysteine and Related Vitamins, University of Bergen, Norway

**Background.** While optimal nutrition is likely to be important in maintaining bone health, the relative importance of individual nutrients has not been established. Recent evidence suggests that tHcy and related B vitamins may be important.

**Aims.** To examine the relationship between B vitamins, tHcy and markers of bone health in postmenopausal women.

**Methods.** Healthy postmenopausal women (n=49) were categorised into *higher* and *lower* folate status using median serum folate (SF) concentration for the group as a whole. Differences in total body bone mineral density (BMD), biochemical bone markers, tHcy, vitamins B12 and pyridoxal 5-phosphate (PLP) were then examined.

**Results.** Women with lower folate status had significantly higher tHcy and significantly lower PLP. There was no significant difference in BMD or plasma free choline and betaine levels. However, in the fate of lower folate status, bone alkaline phosphatase (BAP) and osteocalcin (Ost) were both significantly increased (generally indicative of greater bone turnover). After controlling for 25 hydroxy-vitamin D

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(25OHD), SF was found to be significantly associated with the biochemical bone markers examined.

**Conclusions.** These findings suggest that folate status may be important in determining bone health.

**Table 1.**

	Low n = 24	High n = 25	P value
SF (ng/mL)	6.24 (1.9)	17.9 (9.5)	.000
BMD (g/cm <sup>2</sup> )	1.10 (0.09)	1.12 (0.09)	.411
tHcy (μmol/L)	10.5 (1.4)	9.430 (3.5)	.045
PLP (nmol/L)	38.2 (15.8)	62.96 (27.7)	.002
Vitamin B12 (ng/L)	408.8 (122.0)	483.32 (157.2)	.107
25OHD (nmol/L)	64.8 (26.8)	82.94 (29.94)	.021
BAP (u/L)	33.43 (10.0)	25.98 (7.5)	.007
Ost (ng/mL)	25.40 (14.6)	18.74 (8.1)	.012
Ctx (ng/mL)	0.70 (0.31)	0.54 (0.30)	.081

Values are represented as mean (SD). Differences between the groups were assessed using independent t-tests ( $p < 0.05$ ). C-terminal telopeptides of type-I collagen, Ctx.

## P094

### ASSOCIATION OF SNPS IN HOMOCYSTEINE-RELATED GENES AND RISK OF GASTRIC CANCER: A CASE-CONTROL STUDY

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Gastric cancer is one of the leading cancers in the world and has a low five-years survival rate. Several risk factors are known for gastric cancer, including smoking and infection with *H. pylori*. The role of polymorphisms in genes involved into homocysteine and folate metabolism and carcinogenesis is only partly understood. An association has been reported between MTHFR 677TT and colon cancer. Recently, polymorphisms have also been related to gastric cancer.

Patients with gastric cancer (n=106) were recruited between 1997 and 2002 in the University Hospital Magdeburg. All gastric cancers were histologically confirmed. 28 cases were defined as cancers of the cardia region and 78 were defined as non-cardia. Apparently healthy control subjects (n=106) were recruited by newspaper advertisement during 2001. Exclusion criteria were history of any tumor, clinical diseases of the gastrointestinal tract and use of drugs affecting the GIT. Control subjects were age- and sex-matched with patients. Helicobacter pylori antibodies, serum cobalamin, folate and homocysteine were determined in patients and controls. The following polymorphisms were determined: MTHFR C677T, thymidylate synthase 28 bp tandem repeat (TYMS 28bp), GCP II C1561T, RFC G80A. Neither of these polymorphisms was associated with gastric cancer in the present study. Distributions of genotypes were similar in patients and controls. Among patients with cancer, the RFC 80AA genotype was significantly more present in non-cardia cancers ( $p < 0.03$ ). Allele frequencies of the A-allele were 0.26 in cardia cancers and 0.46 in non-cardia cancers. Results suggest that polymorphisms in genes of the homocysteine metabolism are not associated to the overall risk of gastric cancer. The results of a relation of RFC 80AA to non-cardia cancer must be verified in larger patient groups.

## P095

### DETERMINATION OF TOTAL PLASMA HOMOCYSTEINE: COMPARISON OF A NEW ENZYMIC ASSAY AND A HPLC METHOD

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Over the last two decades a number of studies have shown that moderate and persistent hyperhomocysteinemia is implicated in the development of atherosclerosis and thrombosis.<sup>1</sup> Several methods have been developed for measuring total plasma homocysteine (tHcy), most of them based on HPLC analysis and fluorescence detection. Very recently, a totally automated enzymic assay for tHcy determination (Carolina, Liquid Chemistries Corp, CA, USA), performed on a biochemical analyzer capable of dispensing three reagents and of measuring absorbance at 340 nm, has been introduced. Our aim was to compare the performance of this new procedure and an established home-made HPLC method based on SBD-F derivatization and the use of an internal standard. Since in our Institute tHcy levels are also routinely determined after the methionine loading test, we performed this study in the range: 4.0-60.0 μmol/L. Sodium citrate plasma samples were taken from 96 fasting donors (55 women and 45 men; age: 49±18.7 yr, mean ±SD) and frozen at -80°C until assay. The enzymic assay test is based, after reduction to free HCY, on the following biochemical reactions, catalyzed by CBS, CBL and LD enzymes, respectively.

- 1) serine+Hcy<sup>®</sup> L-cystathionine;
- 2) L-cystathionine<sup>®</sup> hcy+pyruvate+NH<sub>3</sub>
- 3) pyruvate+NADH<sup>®</sup> lactate+NAD

The chromatographic procedure included reduction of disulfide bonds by NaBH<sub>4</sub>, use of cysteamine as internal standard, derivatization with ammonium-7-fluorobenzo-2-oxa-diazole-4-sulphonate (SBD-F), and fluorescence detection after separation by reversed-phase HPLC. Medians and ranges of tHcy plasma levels (n=96) observed were: 16.1 (3.9-61.8) and 17.5 (4.9-58.3) μmol/L for HPLC and enzymic kit, respectively. Linear regression analysis yielded: enzymic assay=1.044 (±0.546)+1.011(±0.024)HPLC (±SE), r=0.975, Sx/y=2.85 ( $p < 0.001$ ). The within- and between-run reproducibilities of two plasma samples (tHcy levels: 9.9 and 51.6 μmol/L) were: HPLC: 2.0% and 3.8% and, 4.0% and 4.7%, respectively; New assay: 2.3% and 1.4% and, 4.1% and 3.7%, respectively. To conclude the enzymic assay is an appropriate method to determine plasma tHcy both for clinical routine and research application, considering its simplicity of execution not requiring a skilled technical staff.

## References

1. D'Angelo A, Selhub J. Homocysteine and thrombotic disease. *Blood* 1997; 90:1-11.

## P096

**MULTIPLE DETERMINATIONS OF METHIONINE LOADING TEST IN A COHORT OF 266 PATIENTS REFERRING TO DEPT. LABORATORY MEDICINE OF OUR INSTITUTE**Mazzola G, Fermo I,<sup>1</sup> Sampietro F, D'Angelo A*Coagulation Service & Thrombosis Research Unit; <sup>1</sup>Lab. of Chromatographic & Separative Techniques IRCCS H San Raffaele, Milano, Italy*

Methionine-loading test was introduced to stress the Hcy metabolic *transsulfuration pathway*, allowing to detect an impairment due to low vitamin B6 status or to heterozygous cystathionine-synthase defect. Thus in clinical studies methionine intolerance was often investigated in patients with cardiovascular disease to establish a correct vitamin treatment. The procedure involves the determination of total plasma homocysteine (tHcy) in the fasting state and after an oral administration of a standard dose of methionine (0.1 g/kg body weight). Blood specimens were then taken after different fixed time interval which range from 2h to 8h. However, the methionine load test suffers from a lack of standardization so the interpretation is often uncertain. In our clinical routine laboratory post-methionine load tHcy levels were specifically measured after 2h and 6h. The method applied was the fluorescence polarization immunoassay (FPIA) adapted to the IMx analyzer (Abbott Laboratories).

Aim of this study was investigated the response to methionine loading test in a cohort of 266 subjects (104 men and 162 women) recruited consecutively from in- and out-patients referring to the Dept. Laboratory Medicine of our Institute. In the Table the tHcy levels ( $\mu\text{mol/L}$ ) after the methionine intake and fasting state (Delta PML 2h and 6h) were also reported.

**Table 1.**

	Men	Women	P
<b>N</b>	104	162	
<b>Age (yr)</b>	52.4±13.16	47.61 ±14.91	0.003
<b>Fasting tHcy</b>	11.91±5.24	9.15±4.19	0.0001
<b>PML tHcy 2h</b>	22.89±8.0	22.1 ±8.6	Ns
<b>PML tHcys 6h</b>	31.67±10.5	29.26±11.56	Ns
<b>Delta PML 2h</b>	10.98±5.3	12.97±6.20	0.006
<b>Delta PML 6h</b>	19.76±8.10	20.11±9.37	Ns

*t-student test was applied*

Interesting is the distribution of hyperhomocysteinemic (Hhcy) patients: 32 women and 13 men showed both high fasting and Delta tHcy levels while 23 women and 23 men had only Delta PML tHcy levels high out of the normal ranges. The different pattern of changes in homocysteine levels observed in men and women suggest that multiple determinations should be performed to accurately evaluate homocysteine metabolism after an oral methionine intake; furthermore 50% of hyperhomocysteinemic patients are only identified by Delta PML with the following percentages: 25% at 2h and the remaining 75% at 6h.

These findings suggest that post-methionine loading test should be expressed using Delta PML tHcy values, which are influenced to a much lesser extent by fasting tHcy levels than absolute tHcy values.

## P097

**IN VIVO ACTIVATION OF MONOCYTES BY METHIONINE LOADING, EVALUATED BY MICROARRAY GENE EXPRESSION PROFILING**Chu WT,<sup>1</sup> de Jonge R,<sup>1</sup> Visser W,<sup>2</sup> van IJcken WFJ,<sup>3</sup> Lindemans J<sup>1</sup>*<sup>1</sup>Clinical Chemistry; <sup>2</sup>Obstetrics and Gynaecology; <sup>3</sup>Center for Biomics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands*

**Background.** Elevated plasma homocysteine is an independent risk factor for atherosclerosis. Interaction between monocytes and endothelial cell lining is thought to play an important role in this process. We investigated whether the transient *in vivo* hyperhomocysteinemia, induced by a diagnostic methionine loading test, causes monocyte activation at the level of gene expression.

**Methods.** Venous blood was drawn from women with a history of pre-eclampsia (n=22, mean age 33±5 years) after 12 hours fasting and 6 hours after an oral methionine load (0.1 g/kg body weight). At the same points of time, blood was also drawn from controls (n=6, mean age 40±10 years) who did not undergo methionine loading but who followed the same methionine-free diet as the patients. Monocytes were isolated at both points of time by Ficoll and Percoll density gradient centrifugation. Total RNA was isolated with Qiagen RNeasy-columns. Reverse transcription, amplification, labelling and hybridization with home-made human microarrays, containing about 19000 oligo's, were carried out according to standard institutional protocols. Processing of the data and statistical analysis was performed using Rosetta Resolver System V.4.0.

**Results.** So far the data from 6 controls and 11 patients have been analyzed. Ten different genes demonstrate a level of activation from basic to 6 hour post-load homocysteine above the  $p=0.01$  limit of significance. Three of them were also found in the control group, not having been subjected to methionine loading. The latter difference is either accidental or caused by circadian rhythm. From the seven remaining, two have no known biological function, two are related to cell proliferation (MAGEE1, CXX1) and the other three are connected with intracellular signalling processes (GPA1, RIMS1, RARG).

**Conclusions.** Methionine loading of recovered pre-eclampsia patients leads to increased expression of a number of genes but none of these genes can be directly connected to known cell-cell interaction mediators.

## P098

**USE OF METHIONINE-HOMOCYSTEINE ALPHA, GAMMA-LYASES TO DEVELOP AN IN VITRO ENZYMATIC DIAGNOSTIC PANEL FOR HOMOCYSTEINE, CYSTEINE, METHIONINE, AND VITAMIN B6**

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We have developed a panel of *in vitro* diagnostics assays for total homocysteine (tHcy), vitamin B6 (PLP), cysteine (CYS) and methionine (MET) based on specific recombinant (methionine) homocysteine alpha, gamma-lyases (rMETase, rHCYase). The principle of the tHcy assay is that rHCYase produces hydrogen sulfide (H<sub>2</sub>S) from tHcy without interference from CYS or MET. H<sub>2</sub>S is determined using N,N-dibutyl phenylene diamine (DBPDA). We have now automated the enzymatic tHcy assay on the Hitachi 912. 3-deazaadenosine (3-DA), which stabilizes the tHcy concen-

tration in whole-blood, does not interfere with rHcyase and the rHcyase-based tHcy assay. We have developed a homogeneous, nonradioactive, enzymatic assay for plasma vitamin B6 (PLP) using the PLP-dependent rHcyase. PLP is stripped from rHcyase to make an apo-enzyme, with the enzyme's activity dependent on reconstitution with PLP in plasma or serum. The H<sub>2</sub>S, released from Hcy, used as the substrate for reconstituted rHcyase holo-enzyme, is measured spectrophotometrically as above. We have developed an enzymatic tCys assay with the use of rMETase that converts Cys to pyruvate, ammonia and H<sub>2</sub>S. rMETase also converts Hcy to H<sub>2</sub>S. To eliminate cross-reactivity, recombinant S-adenosylhomocysteine hydrolase (rSAHH) is used to convert Hcy in plasma to S-adenosylhomocysteine (SAH). H<sub>2</sub>S, released from tCys by rHcyase, combines with DBPDA and is read at 675 nm (as described above). The assay is linear to 500 micromol/L tCys with a detection limit of 10 micromol/L. The methionine assay is based on rMETase which releases methanethiol (CH<sub>3</sub>-SH) from MET. Methanethiol is combined with DBPDA to form a soluble red-colored complex that can be measured at 500 nm. It is expected that this diagnostic panel of homocysteine metabolism will be more informative than homocysteine itself.

**P099**

**THE EFFECT OF HOMOCYSTEINE LOWERING THERAPY ON INFLAMMATORY MARKERS NEOPTERIN AND CRP IN PATIENTS WITH STABLE CORONARY ARTERY DISEASE. A SUBSTUDY OF THE WESTERN NORWAY B-VITAMIN INTERVENTION TRIAL (WENBIT)**

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*Background.* Immune activation and inflammation play an essential part of atherogenesis. Total homocysteine (tHcy) is an independent risk factor for occlusive vascular disease and is associated with inflammatory markers. The association between homocysteine metabolism and the inflammatory processes is unclear.

*Aims.* We tested the effects of tHcy lowering therapy on inflammatory markers known to be associated with tHcy in patients with coronary artery disease (CAD).

*Methods.* Patients (n=130, 80% males) recruited to WENBIT were randomised into four groups to daily oral treatment with 0.8 mg folic acid/0.4 mg vitamin B-12 or placebo, and with 40 mg vitamin B-6 or placebo, in a 2x2 factorial design. Blood was drawn at baseline and after a mean follow up of 7 months.

*Results.* Median age was 60 years. At baseline, we found median plasma tHcy 10.8 mikromol/L, folate 8.7 nM, cobalamin 361 pM and pyridoxal phosphate (PLP) 28.8 nM, and serum creatinine 92 mikromol/L, neopterin 7.1 nM and CRP 1.7 mg/L. Levels of tHcy were significantly correlated with age (r=0.43, p<0.001), folate (r=-0.27, p=0.002), cobalamin (r=-0.23, p=0.01), creatinine (r=0.40, p<0.001), neopterin (r=0.40, p<0.001) and CRP (r=0.18, p=0.04), but not with PLP (r=-0.11, p=0.2). In a multivariate model including all the above variables, serum neopterin remained significantly associated with plasma tHcy (beta=0.22, p=0.02), whereas CRP was not (beta<0.01, p=0.9). Treatment with folic acid/vitamin B-12 caused a significant reduction in plasma tHcy by 31% (-3.5 mikromol/L, p<0.001), but had no significant effect on serum neopterin (p>0.4) or CRP (p>0.4). Vitamin B-6 treatment neither affected tHcy, nor the

inflammatory markers.

*Conclusions.* The immune activating and inflammatory marker neopterin is strongly associated with plasma tHcy concentrations, but it is not influenced by tHcy lowering therapy. These data suggests that elevated level of tHcy and neopterin may share a common mechanism not involving B-vitamin status.

**P100**

**THE EFFECT OF FOLATE SUPPLEMENTATION ON THE PLASMA HOMOCYSTEINE LEVELS IN HIGH CARDIOVASCULAR RISK SUBJECTS TREATED WITH LIPID-LOWERING DRUGS**

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*Background.* Statins and fibrates are the two most common groups of lipid-lowering drugs demonstrated to be capable of exerting certain beneficial non-lipid effects and influencing plasma total homocysteine (tHcy) levels. Aims. To determine whether folic acid supplementation in a daily dose of 0.4 mg lowers plasma homocysteine levels in the same manner as in the subjects treated with hypolipemic drugs.

*Methods.* Forty-two subjects (29 M, 13 F; mean age 54.4 years) with high risk of coronary artery disease and LDL-cholesterol level > 130 mg/dL were randomized to one of the 2 treatment groups (simvastatin or fenofibrate). Blood was sampled four times: at baseline, after a month-long therapy with either simvastatin 40 mg daily (Zocor, Merck Sharp Dohme, n=20) or micronized fenofibrate 160 mg daily (Lipanthyl Supra, Fournier Laboratoires, n=22), and also after 1 and 2 months of additional 0.4 mg supplementation with folic acid (Folik, Polfa Grodzisk) daily. tHcy, folate and vitamin B12 levels in plasma were subsequently evaluated.

*Results.* At baseline mean plasma tHcy levels were similar in both groups. After a month-long hypolipemic therapy tHcy levels were significantly higher in the subjects treated with fenofibrate, as compared to the simvastatin group (16.48±7.03 μmol/L vs. 12.16±3.03 μmol/L; p=0.002). Two-month supplementation of 0.4 mg folic acid daily decreased tHcy levels more significantly in the group treated with simvastatin than with fenofibrate (mean 10.29±2.70 μmol/L vs. 13.50±5.35 μmol/L; p=0.02). Neither group differed in respect of plasma vitamin B12 levels or folate before (6.94±4.14 ng/mL and 6.08±2.7 ng/mL; for simvastatin and fenofibrate respectively) and after 2 months of folic acid supplementation (17.98±6.37 ng/mL and 16.97±7.9 ng/mL; for simvastatin and fenofibrate respectively).

*Conclusions.* It might be reasonably assumed that 0.4 mg of folic acid daily is too small a dose to prevent hyperhomocysteinemia in the subjects treated with fibrates.

## P101

**SERUM HOMOCYSTEINE CONCENTRATIONS AND PARAOXONASE ACTIVITIES IN GREEK PRESCHOOL AGED CHILDREN**Kriebardis A,<sup>1</sup> Karikas GA,<sup>1</sup> Samara I,<sup>2</sup> Schulpis KH,<sup>3</sup> Papachristodoulou M,<sup>1</sup> Fytou-Pallikari A<sup>1</sup><sup>1</sup>Dept of Medical labs, Technological Educational Institute of Athens (TEI-A); <sup>2</sup>Biochemical Lab., Hospital Sotiria, Athens; <sup>3</sup>Institute of Child Health, Aghia Sophia Children Hospital Athens, Greece

Over the past decade it has been well established in adults that elevated serum homocysteine (tHcy), is associated with increased risk for cardiovascular and thromboembolic diseases. However, only little information is available regarding the levels of homocysteine in healthy children and the possible effect of them. The aim of the present study was to measure homocysteine concentrations in healthy preschool Children, to test for differences in tHcy among sex and age and furthermore to test for correlation with Paraonase activity (PON1) the enzyme that was found to contribute to the antiatherogenic properties of high density lipoproteins (HDL) by metabolizing lipid peroxides.

**Methods.** tHcy levels were measured in 134 randomly selected healthy children (71 boys and 63 girls) aged 4-6 years using fluorescence polarization immunoassay (FPIA) technology and PON1 activity was measured toward paraoxon (diethyl-p-nitrophenyl phosphate) substrate.

**Results.** Mean plasma homocysteine was  $7.71 \pm 2.35$   $\mu\text{mol/L}$ . Twenty-two percent (22%) of girls (14/63) and 7% of boys (5/71) had elevated plasma homocysteine levels  $>10$   $\mu\text{mol/L}$ . Homocysteine concentrations were slightly higher in girls as compared to boys ( $8.20 \pm 2.80$  vs.  $7.29 \pm 1.79$   $\mu\text{mol/L}$ ,  $p < 0.03$ ). We did not find any significant interaction between age and homocysteine levels. Mean paraonase-1 activity was 126.81 U/L. There was no difference in the enzyme activity between boys and girls ( $126.81 \pm 69.99$  U/L vs.  $121.74 \pm 64.78$  U/L). A negative relationship between homocysteine concentration and paraonase activity was found with Pearson's correlation coefficient  $r$  equals to  $-0.27$ .

**Conclusions.** Hcy serum levels may be investigated towards preschool children as a possible effective measure against risk of vascular disease. The role of Paraonase (PON1) is under way for further investigation as the enzyme has also homocysteine thiolactone hydrolase activity, an action that should reduce total homocysteine concentration.

## P102

**S-ADENOSYLHOMOCYSTEINE IS INCREASED IN TISSUES OF DIET-INDUCED HYPERHOMOCYSTEINEMIC RATS**van Oppenraaij Emmerzaal D,<sup>1</sup> Heil SG,<sup>1</sup> De Vriese AS,<sup>2</sup> Blom HJ<sup>1</sup><sup>1</sup>Laboratory of Pediatrics and Neurology, Radboud University Nijmegen, Medical Centre, The Netherlands, <sup>2</sup>Renal Unit, University Hospital Gent, Belgium

**Background.** It is hypothesized that hyperhomocysteinemia (HHcy) causes a high S-Adenosylhomocysteine (AdoHcy), and a lower ratio of S-Adenosylmethionine (AdoMet) and AdoHcy. AdoHcy is an inhibitor of many methylation reactions, including those of DNA, RNA, proteins and lipids.

**Aims.** To determine the impact of HHcy ( $83.8 \pm 34.9$   $\mu\text{mol/L}$ ) on AdoMet and AdoHcy levels in different tissues.

**Methods.** Female Wister Rats were sacrificed and the hearts, livers and kidneys were quickly frozen in N<sub>2</sub>. Approximately, 25 mg of tissue was sonicated in PBS. AdoMet and AdoHcy were measured in heart, liver and kid-

ney from control rats and diet induced HHcy rats using solid phase extraction (SPE) and tandem mass spectrometry (MS/MS).

**Results.** The concentrations (nmol/mg protein) of AdoMet and AdoHcy are shown in Table 1.

**Conclusions.** Diet induced HHcy resulted in significantly increased AdoHcy levels and significantly lower AdoMet: AdoHcy ratios in all tissues of HHcy rats compared to control rats. This observation was more profound in heart than in liver and kidney. These results support the hypothesis that HHcy increases AdoHcy and disturbs the AdoMet: AdoHcy ratio, which may impair methylation reactions.

Table 1.

	Controls (n=8) Mean±SD			HHcy (n=8) Mean ± SD		
	heart	liver	kidney	heart	liver	kidney
AdoMet	0.39±0.01	0.57±0.07	0.55±0.06	0.42±0.14	1.12±1.53	0.63±0.10
AdoHcy	0.015±0.005	0.15±0.02	0.061±0.019	0.059±0.089	0.32±0.28	0.15±0.17
AdoMet:AdoHcy	27.5±7.92	3.79±0.72	9.82±3.18	13.8±7.05	2.88±0.97	6.09±2.55

## P103

**HIGH LEVEL OF HOMOCYSTEINE INCREASES RISK OF DELIRIUM POST SURGERY IN ELDERLY FOR HIP FRACTURE**Germgard T,<sup>1</sup> Germgard I,<sup>1</sup> Cederbladh G,<sup>2</sup> Djukanovic I,<sup>3</sup> Koch-Schmidt A<sup>1</sup><sup>1</sup>Department of Chemical and Biomedical Sciences, University of Kalmar, Kalmar; <sup>2</sup>Department of Clinical Chemistry, and <sup>3</sup>Department of Orthopedic, County Hospital, Kalmar, Sweden

Postoperative delirium is a common problem in elderly patients undergoing surgical repair of hip fracture. Thus, it is of great importance to identify treatable risk factors associated with postoperative delirium and thereby lessen its adverse impact to optimize outcome. This study examined the relationship between the incidence of delirium and plasma homocysteine. Eighty-six elderly, not demented, patients (71 women and 15 men with an age range of 65-99 years) admitted to the orthopedic clinic of the county hospital in Kalmar, Sweden, for hip fracture were included.

Appearance of postoperative delirium was established according to the Confusion Assessment Method (CAM). Two to three days after surgery and after an overnight fast, venous blood sample was collected and analyzed for plasma homocysteine. 35% (30 subjects) of the patients became confused 2-5 days post surgery. 43% (13) of patients with postoperative delirium had plasma homocysteine levels  $>12$   $\mu\text{mol/L}$  (mean  $\pm$  s,  $19 \pm 4.7$   $\mu\text{mol/L}$ , median = 17  $\mu\text{mol/L}$ ). A positive correlation ( $15.76$ ,  $p(0,01)=11.34$ ) was thus found between plasma levels of homocysteine and appearance of delirium post surgery for repair of hip fracture. The other seventeen patients, who became delirious, had plasma homocysteine levels  $<12$   $\mu\text{mol/L}$ , mean  $\pm$  s,  $7.65 \pm 1.93$   $\mu\text{mol/L}$ , median = 7.4). Of these, eight patients (26%) were supplemented with cobalamin and/or folate.

As much as 37% (32 patients) of the patients had plasma homocysteine levels  $>12$   $\mu\text{mol/L}$  of which 68% (22 patients) had  $>15$   $\mu\text{mol/L}$ . Thus, more than 25% of the patients had moderate or high homocysteinemia.

The conclusion of this study is that homocysteine is a strong risk factor for postoperative delirium after hip fracture repair. More attention about vitamin status in elderly patients should be taken in general, and supplementation with vitamin B<sub>6</sub>, folate, and vitamin B<sub>12</sub> may counteract postoperative delirium and thereby optimize outcome.

## P104

**DETECTION OF METHYLENETETRAHYDROFOLATE REDUCTASE GENE MUTATIONS AND PLASMA HOMOCYSTEINE IN PATIENTS WITH CARDIOVASCULAR DISEASE**

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It is a well known fact, that elevation of homocysteine in the plasma is a risk factor in the development of cardiovascular disease. It was also established that elevated concentration of homocysteine may be caused by lifestyle and nutrition. One of the reasons of such elevation is often considered in association with polymorphism of 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. The fact that from one hand, the results of previous studies have been contradictory and from the other hand, there is no relevant data of Armenian population, turns the performance of this investigation quite important. The group consists of 29 male and 14 female patients with 18 documented ischemic stroke and 25 myocardial infarction. The PRONTO MTHFR kit in genetic mutation detection was used. Total serum homocysteine was analysed according to the procedure of Axis-Shield homocysteine immunoassay kit. Twenty-one out of 43 (48.0%) investigated patients had C677C (wild type) genotype, 18 (41.9%) - C677T (heterozygotes) and 4 (9.0%) - T677T (homozygotes). Homocysteine level in the plasma was  $12.4 \pm 0.93$ ;  $18.48 \pm 1.87$ ;  $20.22 \pm 2.15$  mmol/L, respectively. These differences in homocysteine level between patients with C677C and C677T and also with C677C and T677T were statistically significant with  $p < 0.01$ . Average level of homocysteine for males in plasma was  $15.03 \pm 1.26$  and for females  $17.0 \pm 1.85$  mmol/L, which is not statistically significant ( $p > 0.1$ ). Presented data showed homocysteine concentration moderate elevation in the groups with genotypes C677T and T677T. This results are in a good accordance with the hypothesis that mutation in MTHFR gene is linked with decrease of MTHFR activity, that in its turn can cause elevation of level of the plasma homocysteine.

## P105

**DISTRIBUTION OF GLUTATHIONE AND RELATED METABOLITES IN DIFFERENT RAT TISSUES**De Graaf-Hess AC,<sup>1</sup> Heil SG,<sup>1</sup> De Vriese AS,<sup>2</sup> Blom HJ<sup>1</sup>*<sup>1</sup>Laboratory of Pediatrics and Neurology, Radboud University Nijmegen Medical Centre, The Netherlands; <sup>2</sup>Renal Unit, University Hospital Gent, Belgium*

**Background.** Oxidative stress became an object of interest during the study of the mechanism via which plasma homocysteine is involved as a risk factor for common disorders. Measurements of the different total and oxidized thiol-compounds in tissue of different organs may contribute in unraveling the underlying mechanism.

**Aims.** To determine total- and oxidized intracellular glutathione, cysteine, cysteinyl-glycine (cys-gly) and gamma-glutamyl-cysteine (glu-cys) in livers, hearts, kidneys and brains of rats.

**Methods.** Female Wistar Rats were sacrificed and the livers, hearts, kidneys and brains were snap frozen in liquid N<sub>2</sub>. Of each tissue 25 to 150 mg was sonicated in a phosphate buffer solution pH 7.2 with or without an excess N-ethylmaleimide. All thiol-compounds were measured by HPLC and fluorescence detection in their reduced form after reduction with sodium borohydride and derivatization with monobromobimane.

**Results.** See Table.

Table 1.

	mean concentration (nmol/mg protein) (n=8)						total glu-cys	total cys-gly
	total glutathion	oxidized glutathion	ox/total ratio (%)	total cysteine	oxidized cysteine	ox/total ratio (%)		
liver	35,0	1,0	3,1	4,7	0,3	5,5	0,5	-
heart	19,1	0,8	4,6	2,7	0,2	6,7	1,3	-
kidney	0,4	0,1	33,6	34,9	1,2	3,4	-	-
brain	2,5	0,2	15,5	15,5	0,3	1,7	0,2	0,8

**Conclusions.** Total glutathione and total cysteine showed the highest concentrations of the thiol containing small molecules in all tissues, but there were great differences of these amounts between the tissue types. Tissue with a high total glutathione concentration had a low total cysteine concentration and vice versa. We like to hypothesize that cysteine may be a surrogate for glutathione especially under low glutathione conditions.

## P106

**DETERMINANTS OF S-ADENOSYLMETHIONINE AND S-ADENOSYLMETHIONINE IN SENIORS**

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**Background.** There is interest in determining whether abnormalities of serum S-adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) play a role in the harmful effects of hyperhomocysteinemia.

**Aims.** We studied demographic and biochemical correlates in seniors in order to understand relationships with SAM, SAH and homocysteine (tHcy).

**Methods.** 149 seniors, 81% female, 30% African-American, mean age 76 yr, had demographic and biochemical variables tested. Serum SAM and SAH were measured by stable isotope dilution liquid chromatography/mass spectrometry.

**Results.** Subjects older than the mean age had higher methylmalonic acid (MMA) ( $p = 0.002$ ) and tHcy ( $p = 0.024$ ) but creatinine (cr), SAM, SAH, ratio, B12 and folate were not different. SAH trended lower in females. Although, folate and MMA were lower in African-Americans, SAH was not different. In contrast, SAM was 78% lower ( $p = 0.001$ ). Multivariate analysis showed that SAM, methylcitrate and cr were the only predictors of SAH. SAM was dependent on MVI use, SAH and sex. tHcy was predicted by B12, MMA, methylcitrate and high cr. MMA was predicted by B12, methylcitrate, SAH, SAM, and sex but not cr or tHcy. There were 45 subjects with elevated MMA and 24 had high cr > 1.2 mg/dL. MMA, B12 and folate were not different in those with high cr, in contrast, to SAM and SAH which were 1.6 and 1.75-fold higher ( $p = 0.005$  and  $0.003$ ). Subjects with normal MMA were randomized to oral B12, 0,25 or 100 mcg/day. No biochemical changes were seen after 0 and 25 mcg, but tHcy fell 7% ( $p = 0.025$ ) and SAM increased slightly ( $p = 0.003$ ) with 100 mcg. B12, 1000 mcg in those with high MMA decreased SAH by 19% ( $p < 0.001$ ), MMA by 47% ( $p < 0.001$ ) and tHcy 26% ( $p < 0.001$ ).

**Conclusions.** 1. Serum SAM and SAH are mutually predicted but otherwise have different correlates in seniors. 2. African-Americans may have lower serum SAM. 3. SAH is highly influenced by cr but can be lowered by B12 treatment of deficient subjects.

## P107

**CASE-CONTROL STUDY OF THE ASSOCIATION OF MATERNAL HCYS LEVELS AND RISK OF HAVING A DOWN SYNDROME CHILD**

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It has been suggested that chronic folate/methyl deficiency affecting normal cellular DNA methylation could increase the risk of having a child with Down Syndrome (DS). Up to date several population studies analyzing the genetic background of folate metabolism have been performed but very few, if any, analysing the biochemical phenotype. In this way the aim of this work has been to analyse the biochemical and genetic factors of the cobalamin/folate metabolism to provide insight on the association of this pathway with the risk of having a child with DS. We have statistically analyzed, in a case-control design, the frequency of three non-synonymous SNPs in genes encoding two proteins involved in the folate and methyl group metabolism (polymorphism 677C>T and 1298A>C in the MTHFR protein and 66A>G in the MTRR protein). We have included in the analysis, in a cohort of control and case mothers, the levels of homocysteine (Hcys) as biochemical marker. The results show that Hcys levels are significantly higher among case mothers than among control mothers ( $p < 0.05$ ). In control mothers plasma Hcys levels were  $14,03 \pm 5,45 \text{ } \mu\text{mol/L}$  ( $n=90$ ) while in case mothers they were  $16,59 \pm 7,89 \text{ } \mu\text{mol/L}$  ( $n=91$ ). We have not observed a maternal age effect for Hcys levels neither a genotype relationship. Analysis of combined genotypes MTHFR C677T/A1298C and MTRR A66G showed that in all analysed genotypes case mothers had higher levels of Hcys than control mothers, with the exception of mothers bearing the T genotype in both alleles. This could be explained because mothers with TT genotype and high Hcys levels have an increased risk of spontaneous abortions during early pregnancy as has been described previously. We have also investigated the relationship both between the genotype and Hcys levels and fetal viability analyzing the frequency of previous pregnancy losses. Our data suggest that the maternal Hcys levels could be affecting fetal viability. In addition, the mothers with previous miscarriages exhibited 15 fold risk to have a child with DS if their Hcys levels are higher than  $13 \text{ } \mu\text{mol/L}$ . In summary our results suggest that high Hcys levels, due to the functional SNP analysed or to other environmental factors (nutrition, lifestyle) could be an independent risk factor for having a child with DS.

## P108

**ENDOGENOUS ANDROGENS LEVELS AND PLASMA HOMOCYSTEINE IN MEN**

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Plasma tHcy levels rise with age in both sexes. Plasma tHcy levels are higher in men than women, which has led to the suggestion that sex hormones may influence tHcy level. A hormonal effect on tHcy levels is supported by studies showing that estrogen plus progestin therapy and tamox-

ifen reduce tHcy concentrations in postmenopausal women. In addition, a study in transsexual males and females showed that tHcy levels decrease after estrogens antiandrogen administration to male (transsexual) subjects. The aim of present study was to determine the association of endogenous testosterone (total, bioavailable), total estradiol, DHEAS levels and fasting plasma tHcy across a wide age range in relatively healthy men. Testosterone (total, bioavailable), sex hormone binding globulin (SHBG), estradiol, DHEAS, and fasting tHcy levels were measured in population based sample of randomly selected men from inhabitants of Utrecht. Anthropometric characteristics were measured and standardized questionnaires were completed. The association serum sex hormone levels and plasma tHcy was examined using multiple linear regression analyses, adjusted for age, diabetes mellitus, smoking, average visceral fat, alcohol intake, prevalent cardiovascular disease and SHBG. No adjustments were initially made for hypertension, as this variable may act as intermediate factors for tHcy effect. Interaction of androgens levels with age, smoking, diabetes mellitus, and CVD were tested using an interaction terms, which were added to the multivariate model. Multiple adjusted linear regression analysis showed that testosterone (total, bioavailable), DHEAS and estradiol levels were not associated with plasma tHcy ( $R=0.075$ ; 95% CI -0.007; 0.158), ( $R=0.077$ ; 95% CI -0.007; 0.158), ( $R=0.034$ ; 95% CI -0.051; 0.119) and ( $R=0.031$ ; 95% CI -0.041; 0.103) respectively. The interaction between age, diabetes mellitus, smoking, and CVD was not statistically significant. Our results do not support any direct role for androgens in the regulation of plasma tHcy level in healthy men.

**Table 1. Regression coefficients resulting from use of log of total homocysteine level as dependent variable and quartiles of endogenous sex hormone levels as independent variable in a study of 400 men.**

	Regression coefficient ( $\mu\text{mol/l}$ ) *	95% CI
Total testosterone (nmol/l)		
(1) 0.24-14.90	Ref	-
(2) 14.90-18.20	0.021	(-0.052; 0.093)
(3) 18.20-22.00	0.048	(-0.026; 0.122)
(4) 22.00-39.60	0.075	(-0.007; 0.158)
Bioavailable Testosterone (nmol/l)		
(1) 0.80-6.70	Ref	-
(2) 6.70-7.78	0.021	(-0.051; 0.093)
(3) 7.78-9.37	0.049	(-0.025; 0.123)
(4) 9.37-16.33	0.076	(-0.007; 0.158)
Total E2 (pmol/l)		
(1) 20.00-75.00	Ref	-
(2) 75.00-85.00	0.057	(-0.016; 0.129)
(3) 85.00-105.00	0.080	(0.007; 0.152)
(4) 105.00-205.00	0.034	(-0.038; 0.106)
DHEAS ( $\mu\text{mol/l}$ )		
(1) 0.20-4.20	Ref	-
(2) 4.20-6.30	0.055	(-0.016; 0.127)
(3) 6.30-8.90	-0.083	(-0.011; 0.155)
(4) 8.90-21.00	0.031	(-0.041; 0.103)

\*Estimates are adjusted for age, diabetes mellitus, smoking, average visceral fat, alcohol intake, prevalent cardiovascular disease and SHBG. †Coefficient of linear regression: change in tHcy, per SD change in sex hormone levels.

## P109

**A LABORATORY-ALGORITHM WITH HOMOCYSTEINE AS THE PRIMARY ASSAY PARAMETER REDUCES THE COST OF INVESTIGATION OF FOLATE AND COBALAMIN DEFICIENCY**

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**Background and Aims.** Analyses of total homocysteine (tHcy) and to some extent methylmalonic acid (MMA) have become increasingly used in Sweden, primarily for investigating folate and cobalamin deficiency. This has led to increasing costs for diagnosis and lab testing since the clinicians still order the established tests, cobalamin and folate. The purpose of this study was to evaluate whether or not a laboratory-algorithm with tHcy as the primary test-parameter, followed by vitamin analyses only when tHcy is above a certain decision limit, could reduce the expanding cost of diagnosing folate and cobalamin deficiency.

**Methods.** For the analysis we used laboratory statistics from two counties in Sweden collected during 2003. We also compared the profile of test parameters in Swedish counties and related these to medical prescription of folate and cobalamin in the same counties. All data was obtained from the publicly available records by courtesy of the respective institutions.

**Results and conclusions.** Correlation between resources spent upon tests and upon treating these deficiencies was poor, and a laboratory-algorithm based upon initial analysis of tHcy, instead of conventional clinical requests for all parameters, could save about 30% of laboratory costs. A typical annual saving in any of these counties (about 260,000 inhabitants) implementing this algorithm would be about 100,000 Euro.

## P110

**IMPAIRMENT OF HOMOCYSTEINE METABOLISM IN PATIENTS WITH RETINAL VASCULAR OCCLUSION AND ISCHEMIC OPTICUS NEUROPATHY**Stanger O,<sup>1</sup> Weger M,<sup>2</sup> Obeid R,<sup>3</sup> Temmel W,<sup>2</sup> Schmut O,<sup>2</sup> Herrmann W<sup>3</sup>*<sup>1</sup>Cardiac Surgery, Cardiovascular Research, Private Medical University Salzburg, Austria; <sup>2</sup>Clinic of Ophthalmology, Medical University Graz, Austria; <sup>3</sup>Clinical Chemistry/Laboratory Medicine, University of Homburg, Germany*

**Background.** Non-arteritic ischemic optic neuropathy (NAION) and retinal vascular occlusion (RVO) are common causes for visual impairment. We have previously reported on the role of hyperhomocysteinemia as a risk factor for vascular eye diseases including NAION and RVO.

**Aims.** The present study investigated whether the determination of metabolites provides more accurate information on the underlying mechanism of impaired homocysteine (Hcy) metabolism in patients with ocular vascular disease.

**Methods.** 51 patients with RVO and NAION (mean 75,7 years) were included in the present retrospective study, if plasma Hcy concentrations were higher than 12  $\mu\text{mol/L}$ . Hcy, creatinine, cystathionine, methylmalonic acid (MMA), folate, vitamin-B12 and B6 concentrations were measured.

**Results.** In absence of folate deficiency, renal function (within normal creatinine levels!) was the most important determinant of Hcy, MMA und cystathionine. Significant correlations were found for all according to creatinine >medi-

an (>106,1  $\mu\text{mol/L}$ ). Mild renal impairment was sensitively indicated by cystathionine ( $R=0,561$ ,  $p<0,001$ ) which also correlated best with Hcy ( $R=0,497$  and  $0,468$ , resp., both  $p<0,001$ ). **Conclusions.** Cystathionine is a very sensitive marker for minor renal dysfunction (within normal creatinine levels), which explained most of the Hcy concentrations in this cohort of patients with ocular ischemic vascular disease. Renal impairment may be underestimated as compared to vitamin deficiencies as cause for homocysteine elevation.

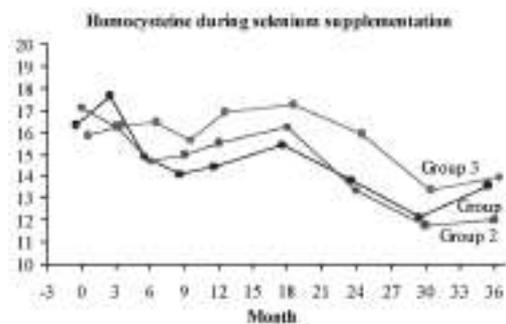
## P111

**THE INFLUENCE OF SELENIUM SUPPLEMENTATION ON PLASMA HOMOCYSTEINE AND MALONDIALDEHYDE CONCENTRATIONS IN ELDERLY HUMANS**Tallova J,<sup>1</sup> Kvciala J,<sup>2</sup> Kralikova M,<sup>1</sup> Tomandl J,<sup>1</sup> Hill M<sup>2</sup>*<sup>1</sup>Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno; <sup>2</sup>Institute of Endocrinology, Prague, Czech Republic*

**Background.** Elevated homocysteine is a risk factor for many pathological states including oxidative stress. The biologically active form of selenium (selenocysteine) is a component of selenoproteins, such as enzymes with antioxidant activity. It is associated with the protection against cardiovascular disease and oxidative stress. A previous study mentioned an inverse relation of serum selenium with plasma homocysteine.

**Aim of the study.** The purpose of this study was to determine the influence of a long-lasting selenium supplementation on the blood selenium concentration, plasma homocysteine, other related thiols and malondialdehyde - the marker of lipoperoxidation, and to search for links between all measured parameters.

**Materials and Methods.** The study sample comprised 75 elderly healthy people (74  $\pm$ 5 years). These have been divided into three groups according to the amount of Se supplementation: group 1 (100 micrograms yeast bound Se/day), group 2 (50 micrograms Se/day) and group 3 (placebo). The supplementation was carried out over a period of 3 years. The analysis of thiols and malondialdehyde was performed by HPLC/FD. The selenium concentrations were measured using neutron activation analysis.



**Results.** The temporal fluctuations of all analyte levels are statistically significant, but without relation to the selenium supplementation. Additionally, the levels of homocysteine ( $r=-0,190$ ,  $p<0,0001$ ) and malondialdehyde ( $r=-0,162$ ,  $p=0,0005$ ) displayed a weak negative correlation with selenium, based on the analysis of all measurements ( $n=462$ ) during the study. These correlations, though weak, are statistically significant due to the high total number of measurements.

**Conclusions.** We found no direct influence of selenium supplementation on the concentrations of the measured analytes, although there were negative correlations between selenium, homocysteine and malodialdehyde when analyzing all measurements. We therefore do not consider selenium as a potential factor for lower homocysteine levels.

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## P112

### HOMOCYSTEINE, FOLATE AND VITAMIN B12 IN SEMINAL PLASMA

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**Background.** Elevated plasma homocysteine is an independent risk factor for the development of many pathological states, but at present, there is no information about its concentration in seminal fluid. It is also well known that folate plays an important role in the reproduction and that the treatment with folate antagonists impairs the reproductive functions in men. A previous study found that low seminal plasma folate concentrations are associated with low sperm density and sperm count and that folate concentrations in the seminal fluid correlate with blood plasma folate and homocysteine. We did not find any data about seminal concentrations of vitamin B12.

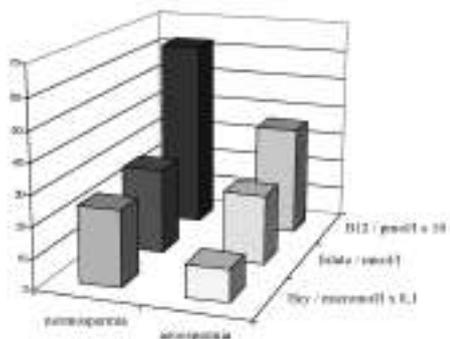
**Aim of the study.** was to measure and determine the concentrations of homocysteine, vitamin B12 and folic acid in the seminal fluid of males with normospermia and azoospermia and to find possible correlations between both groups.

**Materials and Methods.** 26 males (27±5 years old) have been divided into 2 groups according to semen analysis: group I - normospermia and group II - azoospermia. The analysis of homocysteine was performed using HPLC/FD, the analysis of vitamins using the electrochemiluminescence immunoassay.

**Results.** The homocysteine and vitamin B12 concentrations in group I were significantly higher than in group II ( $p=0.0002$  and  $p=0.0028$ , respectively). We found the same relation for folate, but the difference was not statistically significant ( $p=0.1712$ ). We did not find any correlations between age and the measured parameters.

**Conclusions.** The study provides the first evidence of homocysteine in seminal fluid, and a new analytical method has been developed. Lower levels of homocysteine in the group with azoospermia may indicate decreased metabolic activity in the testes of these men. We are not able to explain the metabolic consequences between low homocysteine and low levels of vitamins involved in the homocysteine metabolism.

Homocysteine and vitamin concentrations of normospermia and azoospermia men



## P113

### HYPERHOMOCYSTEINEMIA AS DIAGNOSTIC CRITERION FOR VITAMIN B DEFICIENCY IN INSTITUTIONALIZED ELDERLY

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**Background.** Hyperhomocysteinemia is an accepted risk factor for cardiovascular disease, and possibly also for cognitive impairment and dementia. It has also been proposed as a marker of the status of the B vitamins which are implicated in the metabolic circle. Therefore, especially in the elderly, it is important to know the prevalence of high homocysteine (tHcy) levels and the influence that B vitamins have on them.

**Material and Methods.** Two-hundred and eighteen elderly of both sexes, aged 60-105, living in an Elderly Home in Granada (Spain), were screened for serum folate, red blood cell (RBC) folate, serum cobalamin (Abbott, IMx), holotranscobalamin II (holo-TC II) (HoloTC RIA, Axis-Shield), Methylmalonic Acid (AMM) (MS-GC), total pyridoxine (B6)(HPLC) and tHcy (Abbott, IMx).

**Results.** Hyperhomocysteinemia (tHcy  $12 \geq \mu\text{mol/L}$ ) was detected in 80.7%. Serum folate deficiency was severe ( $< 4 \text{ ng/mL}$ ) in 19.3% and moderate (4-7 ng/mL) in 43.1%. In 14.2% of the elderly RBC folate was  $< 175 \text{ ng/mL}$  and in 61.0% it was between 175-400 ng/mL. Vitamin B12, measured in serum ( $< 200 \text{ pg/mL}$ ), was deficient in 17.4%, but if  $\geq$  measured as Holo-TC ( $< 45 \text{ pmol/L}$ ), deficiency ranges up to 36.7%, AMM was high ( $\geq 300 \text{ nmol/L}$ ) in 42.7%. Vitamin B6 ( $< 20 \text{ } \mu\text{g/L}$ ) was low in 51.4%. In order to identify the factors that could predict tHcy levels, a multiple regression analysis was performed. Best results corresponded to the combination of log serum folate and log AMM, which gives values of  $R > 0.5$ . If analysed independently, the highest correlation is with log serum folate ( $r = -0.290$ ), followed by RBC folate ( $r = -0.263$ ), holo-TC ( $r = -0.228$ ), log B12 ( $r = -0.175$ ) and log B6 ( $r = -0.078$ ).

**Conclusions.** There is a high prevalence of vitamin B deficiency and hyperhomocysteinemia in the studied population. Our data confirm the influence of these vitamins, especially folate, on tHcy levels, but hyperhomocysteinemia can not be used as the only diagnostic criteria to detect subclinical vitamin deficiency in elderly people, especially to detect vitamin B12 and vitamin B6 deficiency.

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## P114

**A PROINFLAMMATORY STATE IS ASSOCIATED WITH HYPERHOMOCYSTEINEMIA IN THE ELDERLY POPULATION**

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Elevated levels of homocysteine may reflect increasing age, male sex, renal failure, vitamin deficiencies (low folate, vitamin B12 or vitamin B6), or inherited abnormalities of enzymes involved in the methionine metabolism. Aim of this study was to determine in a large population based sample whether hyperhomocysteinemia is associated with high circulating levels of inflammatory markers. We studied 586 men and 734 women randomly selected from the people living in two sites in the surrounding of Florence, Italy. In the InCHIANTI population homocysteine, folic acid, vitamin B6 and B12 levels were 13.7 (5.4-95.9)  $\mu$ mol/L, 6.3 (0.7-45.3) nmol/L, 25.3 (0.4-689.2) nmol/L and 285 (25.2-1488.1) pmol/L respectively. Dietary vitamin B6 and folic acid intakes were strongly ( $p < 0.0001$ ) and inversely associated with homocysteine levels, as well as serum levels of folic acid vitamin B12 and vitamin B6. Interleukin 1- receptor antagonist (IL-1ra) and interleukin-6 (IL-6) serum levels were significantly ( $p < 0.001$ ) and positively associated with homocysteine plasma levels also after adjustment for creatinine levels, total energy, lipid, alcohol, and folic acid intakes, as well as serum levels of folic acid, vitamin B12, vitamin B6. Compared to participants in the lowest IL-6 tertile, those in the highest tertiles had higher risk of having high homocysteine (homocysteine  $> 30 \mu$ mol/L) (OR=2.6; 95% CI 1.1-5.6;  $p = 0.024$ ) or homocysteine in the range 15-30  $\mu$ mol/L (OR=1.6; 95% CI 1.2-2.2;  $p = 0.0014$ ). This study provides data about the complex relationship between homocysteine, the pattern of inflammatory mediators, vitamin intakes and vitamin levels in a large population-based study, in the InChianti Study. Interleukin-6 and interleukin-1ra, but not other markers of inflammation, were found to be independent predictors of homocysteine plasma levels, in addition to the vitamins involved in methionine cycle.

## P115

**HOMOCYSTEINE, ASYMMETRIC DIMETHYL ARGININE (ADMA), AND SEMICARBAZIDE-SENSITIVE AMINE OXIDASE ACTIVITY (SSAO): NEW MARKERS OF OXIDATIVE STRESS IN ACUTE CORONARY SYNDROMES**

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High levels of Homocysteine (Hcy) are a well-established risk factor for atherosclerotic disease. One of the mechanism evoked to support its pathogenetic role in atherosclerosis is the induction of an oxidative stress, but *in vivo* data are lacking. *Ex vivo* reports documented an inhibitory effect of high levels of Hcy on the enzyme dimethylarginine dimethylaminohydrolysis (DDAH) which converts the Asymmetric dimethyl arginine (ADMA) in citrullin. Elevated levels of ADMA, the most potent endogenous inhibitor

of the nitric oxide synthase activity, are markers of endothelial dysfunction and are found elevated in patients with atherosclerosis. The semicarbazide-sensitive amine oxidase (SSAO) is a tissue-bound amine oxidase activity highly expressed in the vasculature which determines an endothelial dysfunction through the production of cytotoxic aldehydes and hydrogen peroxide. Elevated SSAO levels have been reported in the metabolic syndrome and in cardiovascular diseases, even if their pathological significance remains to be completely established. The aim of this study was to evaluate Hcy, ADMA levels and SSAO activity in patients with acute coronary syndromes (ACS). Seventy-five patients (age 62.6  $\pm$  11.6; 70 M/5 F) with ACS (51 AMI, 24 UA) referred to the Cardiologic Care Unit of the University of Florence and 75 controls (age 61  $\pm$  9.8; 69 M/6 F) were enrolled. SSAO activity, ADMA and Hcy levels were significantly higher in patients than in controls (31.6  $\pm$  4.6 pmol/mg/h vs 0-2 pmol/mg/h; 1851.58  $\pm$  254.65 pmol/mL vs 520  $\pm$  360 pmol/mL and 22.7  $\pm$  18.4 micromol/L vs 12.3  $\pm$  10.5 micromol/L). Hcy levels significantly correlated with SSAO activity ( $r = 0.57$ ;  $p = 0.001$ ) and with ADMA ( $r = 0.49$ ;  $p < 0.005$ ). In addition, ADMA positively correlated with the extension of necrosis in AMI patients ( $r = 0.46$ ;  $p < 0.05$ ).

This is the first *in-vivo* demonstration that Hcy significantly correlates with ADMA levels in ACS, supporting previous *ex-vivo* data. Furthermore, we found another possible factor which might play a role in the oxidative stress generated by hyperhomocysteinemia. The pathways by which ADMA and SSAO are involved in the oxidative damage caused by HhyperHcy seem to be independent

## P116

**VITAMIN SUPPLEMENTATION NEEDED AMONG ADULTS WITH PHENYLKETONURIA**

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Phenylketonuria (PKU) is caused by an autosomal recessive deficiency of the enzyme phenylalanine hydroxylase leading to a failure to convert phenylalanine to tyrosin. To avoid irreversible neurological damage because of increased phenylalanine, treatment is instituted rapidly after birth. We examined 31 adult PKU patients living on a less protein-restricted diet. 24 patients were only supplemented by essential large, neutral amino acids without vitamins and minerals added, and 7 patients got no supplements at all. Theoretically, these PKU patients had an increased risk of developing vitamin B12 deficiency because animal products are the source of vitamin B12. Besides laboratory ( $n = 31$ ) tests we obtained clinical information ( $n = 30$ ) and detailed information on food consumption ( $n = 28$ ). More than one fourth of the patients had biochemical signs of vitamin B12 deficiency. In spite of a normal folate status, 9 (29%) patients had a plasma homocysteine above 12 micromol/L. These findings might be caused by the fact that according to the food questionnaires 11 (39%) patients received less than the recommended daily vitamin B12, and 20 (71%) patients received less vitamin B6 than the recommended. A significant association was found between reduced plasma vitamin B12 and reduced vitamin B12 intake ( $p = 0.04$ ). 11 patients took a vitamin pill daily, and these patients had a significantly lower plasma homocysteine as compared to the rest. In conclusion the present study suggests that adult PKU patients were at increased risk to develop hyperhomocys-

teinaemia and vitamin B12 deficiency. Considering the risks, costs and potential benefits, daily vitamin supplementation and essential large, neutral amino acids supplied with vitamin B12 seems justified in these patients.

### P117

#### PLASMA HOMOCYSTEINE LEVELS IN PATIENTS WITH METABOLIC SYNDROME

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**Background.** The Metabolic Syndrome (MS) has been shown as an important cluster of risk factors for cardiovascular disease. The mechanisms causing MS are slightly understood, but insulin resistance is thought the major cause. The NCEP and ATP III guidelines also suggest a working definition of the MS that includes the presence of at least 3 of the following characteristics: abdominal obesity (waist circumference >102 cm in men and >88 cm in women), elevated triglycerids (>150 mg/dL), reduced levels of HDL-cholesterol (<40 mg/dL in men and <50 mg/dL in women), high blood pressure (>130/85 mmHg), and high fasting glucose (>110 mg/dL).

**Aims.** This is an observational study evaluating the possible presence of high plasma homocysteine levels in patients with MS.

**Patients and Methods.** Our study involves 47 patients with MS (22 men, age: 34-72 years; 25 women, age: 36-70 years). Patients with type 2 diabetes or smokers were excluded by the study. Plasma homocysteine, triglycerids, HDL-cholesterol and glucose levels were determined by automatic analyzer for biological fluids assay. Body weight was measured to the nearest kilogram, and height to the nearest centimetre. Blood pressure and waist circumference were determined in all patients.

**Results.** We found that in the 47 cardiopatic patients, 36 subjects (76,6%) show both MS and high plasma homocysteine levels, and only 11 subjects (23,4%) show MS with normal plasma homocysteine levels.

**Conclusions.** We can establish that the MS is associated with cardiovascular disease especially in relationship with high plasma homocysteine levels.

### P118

#### COMPARATIVE EFFECT OF DAILY AND WEEKLY FOLIC ACID SUPPLEMENTATION ON PLASMA TOTAL HOMOCYSTEINE

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Fasting plasma total homocysteine (tHcy) concentrations are associated with increased risk of cardiovascular disease. Daily supplementation with 400µg folic acid decreases tHcy concentrations. The aim of this study was to compare the homocysteine-lowering effect of folic acid supplements taken daily or weekly. One hundred and four participants with a tHcy concentration greater than 9 µmol/L were randomised to receive a placebo tablet daily, or supplements containing 400 µg folic acid taken once per day, or 2800 µg folic acid taken once per week, for 16 weeks. The mean (SEM) red blood cell folate concentrations, adjusted for baseline values, in the placebo, daily, and once-a-week folic acid groups at the end of the 16-week trial were 702 (51), 883 (50), and 1117 (49) nmol/L, respectively. The overall mean

(95% CI) baseline tHcy concentration of all participants was 10.6 (10.2, 11.1) µmol/L. Supplementation with folic acid for 16 weeks reduced mean (95%CI) tHcy concentrations by 15.0 (9.3, 21.1)% and 11.4 (5.7, 17.3)%, in the daily and weekly groups, respectively, relative to the placebo group and adjusted for baseline values. The mean reductions in tHcy in the daily and once-a-week folic acid groups were not different ( $p>0.05$ ). Once-a-week supplementation with 2800 µg of folic acid is efficacious at reducing tHcy in people with slightly raised concentrations.

### P119

#### DETERMINATION OF METHYLMALONIC ACID, HOMOCYSTEINE AND RELATED AMINO ACIDS BY A METHOD BASED ON METHYLCHLOROFORMATE DERIVATIZATION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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**Background.** The combined measurement of methylmalonic acid (MMA) and total homocysteine (tHcy) in serum/plasma is useful in diagnosing and distinguishing between cobalamin and folate deficiency. The simultaneous determination of methionine (Met), cysteine (Cys), serine (Ser) and glycine (Gly) may provide additional information about metabolic abnormalities related to one-carbon metabolism.

**Aims.** The aim of this study was to develop and validate an isotope dilution gas chromatography-mass spectrometry (GC-MS) method for the determination of MMA, tHcy, Met, Cys, Ser and Gly in serum or plasma that is suitable for automated sample workup.

**Method.** Serum or plasma samples (100 µL) were treated with a reductant (dithioerythritol), deproteinized with ethanol, and derivatized and extracted in a single step by addition of methylchloroformate and toluene. All liquid handling was carried out in 96-well (1 mL) microtiter plates using a robotic workstation. The derivatives were analyzed by GC-MS in the selected ion monitoring mode.

**Results.** The assay was linear to 100 µmol/L for MMA and tHcy, and to 1000 µmol/L for Met, Cys, Ser and Gly. Detection limits (signal to noise ratio 5:1) were between 0.03 µmol/L (MMA) and 10 µmol/L (Ser, Cys). The within-day coefficients of variation (CVs) ranged from 0.7 to 3.6% (n=20) and the between-day CVs from 2.1 to 8.1% (n=20). The recovery was between 79% and 99% for the different analytes.

**Conclusions.** This high throughput assay combines a simple and automated sample preparation with selective and sensitive GC-MS analysis and is well suited for the combined measurement of MMA, tHcy and the related amino acids.

### P120

#### VARIATION OF PLASMA TIOLIC STATE IN MULTIPLE SCLEROSIS VERSUS CEREBROVASCULAR PATIENTS AND HEALTHY SUBJECTS AFTER METHIONINE LOAD

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system but current knowledge indicates that the physiopathologic mechanisms

include both inflammatory and degenerative processes.<sup>1</sup> The nervous system has high metabolic rate and is very rich in oxidizable substrates: catecholamines and polyunsaturated lipids, in addition to DNA. However, the inaccessibility of the brain to biochemical monitoring and the short living feature of ROS hamper the studies on the role of the oxidative stress in MS. Sulfhydryl groups on glutathione and albumin are oxidized by reactive oxygen metabolites, e.g. peroxide, hydrogen peroxide, hydroxyl radical and superoxide anion. An increasingly important area of antioxidant defense is based on sulfhydryl chemistry, owing to the role of sulfhydryl groups in the function of macromolecular structures such as enzymes and cellular membranes. Since altered serum sulfhydryl levels are described in a number of autoimmune-reaction diseases the cellular oxidation-reduction state could provide a biochemical marker of the oxidative stress in MS patients.<sup>2</sup> Moreover, hyperhomocysteinemia has been identified as a risk factor of cerebrovascular disease (CVD) in several studies. Experiments of methionine load are useful to characterise defects in the transsulfuration pathway of homocysteine (Hcy) and its metabolites. In fact, after the methionine load it is observed a considerable increase in the concentration of S-adenosylmethionine and this tend to suppress the remethylation of Hcy, by inhibiting methylenetetrahydrofolate reductase and increases Hcy effects. On the basis of these observations, the aim of the research was to compare plasma distribution of various endogenous thiols (cysteine, cysteinylglycine, homocysteine and glutathione), after oral methionine loading (100 mg/kg bw), in MS and CV patients versus healthy subjects. Results demonstrated that the methionine load caused modification overtime of the redox state of different thiols in both groups. In particular, levels of total cysteine in CV and MS patients were always much higher than controls. Our data suggest that increase of Hcy in plasma after methionine intake may enhance activity of the transsulfuration pathway. It will remain therefore to establish if cysteine increases in CV and SM patients can be an additional element of toxicity of the Hcy.

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P121

**HOMOCYSTEINE AND THEIR METABOLITES IN RHEUMATOID ARTHRITIS IN COMPARISON TO NORMAL CONTROLS UNDER EPIDEMIOLOGICAL VIEWPOINTS**

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**Background.** Rheumatoid arthritis (RA) is characterized by inflammatory and immunological phenomenons. Moderate hyperhomocysteinaemia is now common in autoimmune diseases, e.g., RA. The mechanisms responsible for hyperhomocysteinaemia in RA are not clear. Hyperhomocysteinemia is associated with a risk for atherosclerosis.

**Objectives.** The aim of this study was to investigate the levels of total homocysteine (tHcy) and their metabolites cystathionine (Cysta), methylmalonic acid (MMA), methionine (Met), sarcosine (Sar), dimethylglycine (DMG) and

methyl citric acid (MC) in patients with RA and normal controls (NC) in consideration of sex and hormonal state of the appropriate group.

**Methods.** 76 RA patients and 59 NC were recruited for this study. All patients fulfilled the ACR-criteria of 1987 for RA. All patients were fasting before blood collection. The levels of tHcy and all metabolites were examined using the gas chromatographic-mass spectrometric assay after sample preparation. Statistic evaluations were performed by SPSS 11.5.

**Results.** (see Table below).

**Discussion and conclusions.** In the present study influences of disease, sex and hormonal state on the levels of tHcy and their metabolites were investigated in comparison of RA patients to NC. The investigations and findings show clearly differences based on disease, sex and hormonal state. No difference between RA and NC was found for tHcy. Only Cysta presented a high significant difference. Unlike, significant differences for tHcy could be only shown between male and female RA patients and for female NC in pre- and postmenopausal state. MMA demonstrated only differences in sex and hormonal state events in the RA group. We conclude, that it is important to look not only for tHcy but also for the related metabolites MMA and Cysta, which describe the actual vitamin state. Missed significant differences in tHcy between RA patients and NC may be influenced by other factors than disease itself like disease modifying drugs, NSAIDs and Glucocorticoids. This topic will be included in a further study. It is also important to show, that sex-specific standards and the hormonal state depended standards are important for characterizing differences between an inflammatory disease like RA and NC.

Table 1.

	Met	Sar	DMG	tHcy	Cysta	MMA	MC2	MC1	MC
RA vs. NC	0.000 <sup>1)</sup>	0.000			0.000				
RA and sex		0.000	0.000	0.053					
NC and sex	0.036								
RA vs. male NC	0.001	0.002				0.042			
RA vs. female NC	0.000	0.000			0.000			0.030	
Menopause RA		0.030	0.020			0.026			0.048
Menopause NC		0.024		0.006	0.000			0.044	
Before menopause RA vs. NC			0.002			0.003			
After menopause RA vs. NC		0.000	0.000						

<sup>1)</sup> Results are given as the p value of the Mann-Whitney U-Test for two independent samples; p<0.0001 □ 0.000

## P122

## BETAINE IS A DETERMINANT OF PLASMA LIPIDS IN MAN

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Betaine is a methyl donor that feeds into the methionine cycle and has been recognised as a lipotropic agent for some time. More recently, its role as a determinant of plasma homocysteine has become a new focus of interest. Hyperhomocysteinemia and hyperlipidemia have both been shown to be independent risk factors for the development of atherosclerosis. We therefore investigated the association between plasma betaine and lipids in 502 patients with different severities of coronary artery disease that had undergone clinically indicated angiography. We also investigated whether the presence of the R239Q polymorphism of the enzyme betaine homocysteine methyltransferase (BHMT) would influence the plasma concentrations of its substrate betaine or its product dimethylglycine (DMG). Plasma betaine was strongly determined by sex. The BHMT R239Q polymorphism was not associated with any plasma parameters. However, a multiple linear regression model of plasma parameters demonstrated a significant negative correlation of betaine with triglycerides, total cholesterol, high-density cholesterol and apolipoprotein A1. In this model, there was no independent association of betaine with coronary artery disease. We conclude that the lipotropic action of betaine is reflected in plasma lipoprotein levels and is not independently associated with CAD.

## P123

## HOMOCYSTEINE AND GLUTATHIONE PEROXIDASE 1 ACTIVITY IN ACUTE CORONARY SYNDROMES

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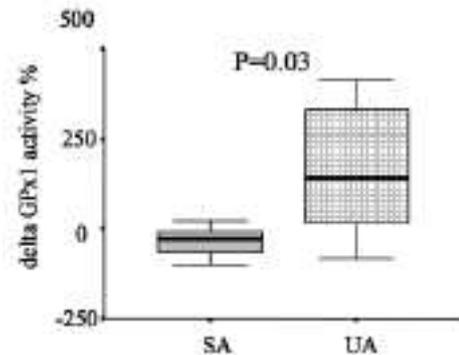
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**Background.** Several lines of evidence point to a key role of increased oxidative stress in acute plaque activation and vascular thrombosis. Aim of this study was to evaluate the redox status of amino thiols and the intracellular glutathione peroxidase (GPx-1) pathway, a major antioxidant during oxidative stress, in acute coronary syndromes (ACS).

**Methods.** We studied 21 ACS patients, who presented to the emergency room within 12 hours after the onset of chest pain (unstable angina, UA) and 19 age- and gender-matched controls with stable angina (SA). Plasma and blood cysteine, cysteinylglycine, homocysteine (Hcy), glutathione and erythrocyte activity of glutathione peroxidase (GPx-1) were analyzed in samples collected at presentation and at discharge, on average 5 days after the onset of symptoms.

**Results.** Plasma reduced Hcy/total Hcy ratio was higher ( $p=0.049$ ) in UA patients than SA controls both at baseline [median (interquartile range) 0.015 (0.008; 0.027) vs 0.01 (0.01; 0.015) respectively] and at discharge [0.012 (0.008;

0.016) and 0.01 (0.01; 0.02) respectively]. No other differences were found in plasma or blood amino thiol concentrations between UA and SA. GPx-1 activity increased in UA patients while it decreased in SA controls and changes at discharge from baseline (delta GPx-1) were significantly larger in UA compared to SA (see Figure below).



**Conclusions.** The higher reduced Hcy/total Hcy ratio, despite similar baseline thiol concentrations, in UA patients and in SA controls points to an association between the clinical equivalents of plaque rupture and an imbalance in homocysteine metabolism. Importantly the increasing activity of GPx-1 in the subacute phase of UA lends further support to the putative role of this enzyme as a defense against oxidative stress in the cardiovascular system. Whether failure of this protective mechanism may adversely impact on outcome in ACS needs further investigation.

## P124

## MOLECULAR CHARACTERISATION OF HOMOCYSTEINURIA IN FIVE ITALIAN PATIENTS WITH CYSTATHIONE BETA-SYNTASE DEFICIENCY

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**Background.** Homocystinuria is a metabolic disorder due mainly to cystathione beta-synthase (CBS) deficiency producing an increased urinary excretion of homocysteine and methionine. Its major clinical manifestations involve ocular, central nervous, skeletal, and vascular systems with high inter-familial and intra-familial phenotype variability.

**Aims.** Our aim was to characterize the CBS mutations in homocystinuric patients to investigate whether mutations in the COOH-terminal of the CBS protein are associated to connective tissue manifestations as hypothesized by Maclean et al (2002). Genetic polymorphisms (MTHFR C677T, MTHFR A1298C, FV Leiden, FII G20210A, MTR A2756G) associated to thrombosis or to homocysteine metabolism were also studied.

**Methods.** We investigated the genotype and the phenotype of 5 Italian homocystinuric patients, two of whom presenting ectopia lentis as major connective tissue feature and three with thrombotic events. The CBS gene was performed by denaturing high performance liquid chromatography (dHPLC) and direct sequencing of the heteroduplexes; the genetic polymorphisms underwent real time fluorescence PCR or NanoChip technology analysis.

**Results.** We found 8 heterozygous mutations: six missense [(R125Q, R336H, A157P, P49L, G307S, I278T), one deletion

(del I429), one splice mutation (IVS 12,+1, G>A)] and one frequent silent polymorphism. Two out of 8 mutations (missense A157P; deletion I429) are novel. Regarding the polymorphisms one homozygous and one heterozygous for MTHFR A1298G and C677T were detected; heterozygous MTR A2756G was found in 2 out of 5 patients. All patients had normal values of Protein C, Protein S and antithrombin, and anti-phospholipid antibodies were absent.

**Conclusions.** We detected 8 out of the 10 expected mutations in our patients, the two missing mutations are probably localized in MTHFR gene, an other gene associated to homocystinuria. In our patients we were not able to confirm the genotype-phenotype correlation proposed by Maclean and coworkers.

### P125

#### SEVERE MYOPIA HERALDING THROMBOEMBOLIC EPISODES

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**Hippocraton** Cystathionine beta-synthase (CBS) deficiency (OMIM#236200) is an autosomal recessive inborn defect of homocysteine (Hcy) metabolism resulting in accumulation of Hcy and methionine (Me) in body fluids. Common clinical features are ectopia lentis, bone abnormalities and thromboembolic episodes which are the usual cause of death. Pyridoxine is the therapy of choice. A patient with CBS deficiency whose first clinical manifestation was severe myopia is presented. The objective of this report is to increase clinical awareness because ophthalmological abnormalities commonly precede vascular episodes whereas early diagnosis and therapy can prevent this potentially lethal complication. The patient is the 10 year old male offspring of unrelated parents who was admitted to the ICU in a comatose state. Brain magnetic resonance imaging revealed thrombosis of the superior sagittal sinus. Eleven days later he developed thrombosis of the left popliteal vein. According to his history he had severe myopia (>6 degrees) from the age of six years. Ophthalmological examination revealed dislocation of both lenses. His father had thrombophlebitis in a lower limb. A screen for thrombophilic factors in our patient was negative but aminoacid analysis revealed increased levels of plasma tHcy (256 µmol/L, nv 3-10) and methionine (181 µmol/L, nv 9-36). The clinical features in combination with the high Hcy and Me levels indicated CBS deficiency. CBS enzyme assay in cultured skin fibroblasts is pending. After 20 days combined vitamin B therapy (B12, B2, B6) and folic acid, tHcy levels dropped from 256 to 32 µmol/L and Me from 181 to 30 micromol/L. Investigation of his family revealed increased tHcy levels in his father and mother (49 micromol/L and 21 micromol/L respectively) and normal levels in his sibling. In conclusion, severe myopia in children should always be investigated for CBS deficiency because early diagnosis can be life-saving. High - risk family members should be investigated as well.

### P126

#### THE MECHANISM OF ABERRANT SPLICING IN CBLE TYPE OF HOMOCYSTINURIA

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The cble type of homocystinuria is a rare autosomal recessive disorder caused by methionine synthase reductase (MTRR) deficiency. The most common pathogenic mutation in the MTRR gene is an intronic substitution c.903+469T>C, which putatively activates an exonic splicing enhancer (ESE) and leads to an insertion of a 140 bp pseudoexon between exon 6 and 7 (Wilson *et al.*, 1999). The aim of our study was to explore experimentally whether the T>C transition activates possible ESEs in intron 6. Firstly, the *in silico* analyses of intron 6 revealed the presence of cryptic splice sites delimiting the pseudoexon and showed that the T>C substitution in overlapping ESE motifs putatively increases their binding affinity for splicing factor SF2/ASF. In summary, *in silico* analyses predict that the c.903+469 T>C substitution increases the signal of ESE motifs, which in turn recruit splicing factors and facilitate the usage of cryptic splice sites for pseudoexon inclusion. As the next step, we analysed the splicing products of a 700 bp portion of intron 6 carrying either C or T in position c.903+469 using exon trapping technique in COS-7 cells. The results clearly showed that only the C-allele yielded the mRNA molecules containing the 140 bp pseudoexon. The proportion of aberrantly and normally spliced transcripts produced from C-allele was approximately equal, demonstrating that the aberrant splicing is not complete. This part of our study demonstrated experimentally that the T>C transition is indeed the culprit of pseudoexon inclusion. To summarise, the *in silico* and functional analyses showed that the most common pathogenic c.903+469T>C allele in the MTRR gene produces mRNA molecules carrying the 140bp insertion via activation of ESE motifs in intron 6.

### P127

#### CAROTID ARTERY ULTRASONOGRAPHIC FINDINGS, LIPOPROTEIN (A), SERUM LIPID AND DIETARY FAT INTAKE PROFILES IN CLASSICAL HOMOCYSTINURIA: EFFECTS OF CHRONIC TREATMENT AND BIOCHEMICAL CONTROL

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The clinical syndrome of classical homocystinuria (HCU), due to cystathionine beta synthase deficiency, includes mental retardation, ectopia lentis, premature osteoporosis and vascular events. Pulmonary embolisms and severe carotid or coronary obstructions causes early morbidity in untreated HCU patients. We assessed the carotid system by ultrasound, lipoprotein (a), fasting serum and dietary lipid profiles in 32 HCU patients (19 males, 13 females; 28 pyridoxine nonresponders) with a mean (range) age of 21.3 (2.7-46.4) years. Nineteen were diagnosed through newborn screening and treatment commenced within six weeks of birth, the remaining 13 were late detected and treated upon diagnosis. Treatment consisted of dietary methionine restriction with cofactor supplementation for pyridoxine nonresponders and pyridoxine, B12, folic acid for responders. There were 491 patient treatment years. A mean of lifetime total homocysteine (tHcy) was calculated for each patient to define the bio-

chemical control. All patients had normal flow and no plaque formation in the common carotid (CC) and internal carotid (IC) arteries (colour Doppler). One patient had raised right sided IC:CC systolic velocity (pulsed Doppler) while another had raised right sided IC:CC systolic and diastolic velocities consistent with 40-59% luminal diameter reduction according to normative data. All but four, had normal Lipoprotein (a) levels of < 30mg/dL. All had normal fasting total cholesterol (normal 3.5-6.5), triglycerides (normal 0.5-2) and a total cholesterol: HDL < 3.5. Range of total dietary fat intake was 22.9-65.7% of total dietary energy. The mean lifetime tHcy ranged from 42-179 micromols/L (normal <15). The patients with abnormal carotid artery findings were poorly compliant to treatment with levels of 128.7 and 149.1 micromols/L and has developed other complications of untreated HCU. This study of 32 chronically treated HCU patients with 491 patient treatment years demonstrates that homocysteine lowering treatment is effective in preventing carotid artery disease, despite imperfect control.

**P128****ASSEMBLY OF CBS MUTANTS AS A DETERMINANT OF CATALYTIC ACTIVITY**

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Decreased or absent activity of cystathionine beta-synthase (CBS) is caused by more than 130 different mutations, however, the underlying molecular mechanisms are only partially understood. Our group has proposed previously that misfolding of CBS mutants may be a common pathogenic mechanism in homocystinuria. In this study we examined whether the presence of correctly folded tetramers is a determinant of residual catalytic activity in a series of 27 patient-derived CBS mutations. Mutants were expressed in *E. coli* at 37°C and 18°C using a pKK derived vector, proportion of tetramers (at least 10% of signal) and aggregates was assessed by western blotting using non-denaturing conditions and catalytic activity was measured by a standard assay. One third of mutants assembled into tetramers already after being expressed at 37°C, an additional one quarter of mutants formed tetramers after expression under folding-permissive conditions (i.e. at 18°C). The ability of enzyme molecules to fold properly seems to be a necessary requirement for their catalytic activity as none of the catalytically active mutants lacked detectable amounts of correctly assembled tetramers. Mutations located in the same domain of the enzyme tend to exhibit common features: i/ mutations in the catalytic site are predominantly aggregated and inactive regardless of the expression conditions; ii/ the majority of mutations in the COOH-terminal portion assemble into tetramers, which are highly active under both expression temperatures; iii/ major impact on mutations in the dimer-dimer interface and heme-binding site was achieved by low temperature expression, which rescued tetramerization and activity in 4 out of 7 mutants. Our data suggest that correct assembly is a necessary but not the only sufficient requirement for catalytic activity of CBS mutants. The study demonstrates that misfolding of mutant enzyme is one of several pathogenic mechanisms in homocystinuria, especially relevant to mutations located close to heme binding site and in the dimer-dimer interface.

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**P129****EVIDENCE FOR A CRITICAL ROLE OF THE MODIFYING SUBUNIT OF GLUTAMATE-CYSTEINE LIGASE IN HOMOCYSTEINE TOXICITY**

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**Background.** Hyperhomocysteinemia (HHcy) is associated with impaired endothelium-dependent vasodilatation. Despite intensive research, the pathophysiology of this association remains conjectural. Some gene-expression studies were performed in the past to unravel the pathophysiology of HHcy, in which generally cultured cells or physiologically irrelevant concentrations of homocysteine were used.

**Aims.** In this study, we performed microarray analysis on aorta of an in vivo rat model of hyperhomocysteinemia to explore the pathophysiology involved.

**Methods.** Female Wistar rats were fed a diet rich in methionine with low levels of B-vitamins to induce HHcy (n=8) or were fed standard rodent chow (n=8) for 8 weeks. RNA was isolated from aorta, amplified and labeled by T7-based RNA amplification, and subsequently hybridized to a 5K oligonucleotide array (Sigma-Genosys). Normalized datasets were analyzed and corrected for multiple testing by significance analysis of microarrays. The results of the microarray analysis were validated by application of real-time quantitative PCR (Q-PCR) using SybrGreen (iCycler IQ, Biorad). Levels of total homocysteine, cysteine,  $\gamma$ -glutamylcysteine, glutathione and cysteinylglycine were measured by HPLC analysis in serum of HHcy and control rats.

**Results.** Nine genes were differentially expressed in aorta of hyperhomocysteinemic rats. The up-regulation of the modifying subunit of glutamate-cysteine ligase (GCLM), a gene involved in glutathione biosynthesis was confirmed by Q-PCR analysis (2.7-fold,  $p=0.03$ ). Application of HPLC analysis showed that total glutathione levels were higher in serum of HHcy rats compared to control rats ( $39.8 \pm 8.6 \mu\text{M}$  versus  $22.3 \pm 3.9 \mu\text{M}$ ), which strongly correlated with GCLM mRNA expression in aorta ( $R_s=0.79$ ,  $p=0.004$ ).

**Conclusions.** We have identified a novel pathway involved in homocysteine toxicity. Our findings suggest that increased levels of glutathione are produced by GCLM to compensate for the adverse oxidative effects of elevated homocysteine on the vascular wall.

**P130****THE -588 C>T SNP IN THE HUMAN GCLM GENE INTERACTS WITH ELEVATED HOMOCYSTEINE LEVELS AND INCREASES THE RISK OF VENOUS THROMBOSIS**

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**Background.** Hyperhomocysteinemia (HHcy) is associated with increased risk of cardiovascular diseases. Recently, we identified a novel pathway in homocysteine toxicity as we demonstrated that the modifying subunit of glutamate-cysteine ligase (GCLM), the rate-limiting enzyme in glutathione synthesis, was up-regulated in aorta of rats fed a high

## Homocysteine Metabolism

methionine/low B vitamin diet. In humans a single-nucleotide polymorphism (SNP) in the promoter region of the GCLM gene (-588 C>T) has been reported, which reduces GCLM mRNA expression under conditions of ambient oxidative stress.

**Aims.** We hypothesized that a hampered up-regulation of GCLM due to this -588 C>T SNP increases the risk of vascular diseases, in particular in the presence of oxidative conditions such as HHcy.

**Methods.** The -588 C>T SNP of the GCLM gene was genotyped by PCR followed by restriction-enzyme analysis in 170 cases with a history of recurrent venous thrombosis and 433 controls from the general population.

**Table 1.**

	total glutathion	oxidized glutathion	ox/total ratio (%)	total cysteine	oxidized cysteine	ox/total ratio (%)	total cys-gly	total glu-cys
<b>liver</b>	35,0	1,0	3,1	4,7	0,3	5,5	0,5	-
<b>heart</b>	19,1	0,8	4,6	2,7	0,2	6,7	1,3	-
<b>kidney</b>	0,4	0,1	33,6	34,9	1,2	3,4	-	-
<b>brain</b>	2,5	0,2	15,5	15,5	0,3	1,7	0,2	0,8

mean concentration (nmol/mg protein) (n=8)

**Results.** The frequency of the -588 TT genotype was higher among recurrent venous thrombosis cases than controls, which led to a slightly increased risk of venous thrombosis (OR 1.33 [0.59-2.90]). Interestingly, interaction analysis demonstrated that this risk increased to more than 2-fold when also homocysteine levels were elevated (OR 2.55 [1.04-6.11]).

**Conclusions.** These findings show that the -588 C>T SNP of GCLM interacts with HHcy, which results in an increased risk of recurrent venous thrombosis. The results of this human study confirm the previously identified role of GCLM in homocysteine toxicity, and point to the involvement of oxidative stress.

## P131

### ERYTHROCYTE ANTIOXIDANT STATUS IN ACUTE AND CHRONIC HYPERHOMOCYSTEINEMIA

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An imbalance in biological red-ox systems, leading to an oxidative stress, has been proposed in the pathogenesis of vascular damage induced by high level of homocysteine (Hcy). In the present study we investigated the erythrocyte antioxidant status (ANTOX) and the erythrocyte resistance to an oxidative stress (RBC-OSR) in 40 subjects with hyperhomocysteinemia (HHcy) compared with 42 with normohomocysteinemia (NHcy). ANTOX was also measured after acute induction of HHcy by methionine oral load (MOL) in NHcy. HHcy subjects had lower erythrocyte vitamin E (vit E-RBC) content [significantly lower only in HHcy subgroup with higher plasma Hcy (Intermediate HHcy, IHHcy)], in spite of normal total plasma vitamin E concentrations. Activities of erythrocyte superoxide dismutase and glutathione peroxidase were significantly reduced in IHHcy subjects, as well. This was significantly decreased in IHHcy compared with NHcy subjects. All ANTOX parameters and RBC-OSR resulted significantly reduced in NHcy after acute induction of HHcy (MOL). These results suggest a level-dependent altered antioxidant status of RBC in the presence of HHcy,

associated with an increased erythrocyte susceptibility to an oxidative stress. Vitamin E content in RBC might be a sensitive parameter for evidencing early oxidative stress and it may play a role in prevention of Hcy-induced damage.

## P132

### POTENTIAL CELLULAR MECHANISMS BY WHICH TDAG51, A MEDIATOR OF ATHEROGENESIS, PROMOTES DETACHMENT-INDUCED PROGRAMMED CELL DEATH

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Hyperhomocysteinemia (HH) is an independent risk factor for cardiovascular disease and accelerates atherosclerotic lesion development in apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice. Previous studies have demonstrated that homocysteine causes endoplasmic reticulum (ER) stress and programmed cell death (PCD) in cultured vascular endothelial cells. However, the cellular factors responsible for this effect and their relevance to atherogenesis have not been completely elucidated. Recently, we have demonstrated that homocysteine induces the expression of T-cell death associated gene 51 (TDAG51), a member of the pleckstrin homology-related domain family, in cultured human vascular endothelial cells. This effect was mimicked by other ER stress-inducing agents, including DTT and tunicamycin. TDAG51 expression was attenuated in homozygous A/A mutant eIF2[alpha] mouse embryonic fibroblasts treated with homocysteine or tunicamycin, suggesting that eIF2[alpha] phosphorylation is required for TDAG51 transcriptional activation. Transient overexpression of TDAG51 elicited significant changes in cell morphology, decreased cell adhesion and promoted detachment-induced PCD. In support of these *in vitro* findings, TDAG51 expression was increased and correlated with PCD in the atherosclerotic lesions from apoE<sup>-/-</sup> mice fed hyperhomocysteinemic diets, compared to mice fed control diet. We have further assessed the effect of TDAG51 overexpression on cytoskeletal organization and structure, and defined the functional domains of TDAG51 that contribute to PCD. Furthermore, we have investigated whether loss of TDAG51 protects cells from ER stress-induced PCD. To better assess the role of TDAG51 in atherosclerotic lesion development and progression, knockout mice deficient in both TDAG51 and apoE have been generated. These findings provide a better understanding of the molecular mechanisms that contribute to the proapoptotic effects of TDAG51 and may provide opportunities for the development of therapeutic modalities useful in the treatment of cardiovascular disease.

## P133

### PLASMA S-ADENOSYLHOMOCYSTEINE CONCENTRATION IS A FUNCTION OF GLOMERULAR FILTRATION RATE IN CHILDREN

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**Background.** Homocysteine is an independent risk factor for vascular disease. Patients with renal disease have increased levels of plasma homocysteine. Previous studies

have shown that plasma S-adenosylhomocysteine is a more sensitive indicator of the risk for atherosclerotic vascular disease and is a more sensitive measure of renal insufficiency than plasma homocysteine. However, in these studies the patients were older and had a variety of other diseases such as diabetes and primary hypertension that are associated with vascular disease and could reduce renal function by involvement of the kidneys

**Aims.** To measure plasma homocysteine and S-adenosylhomocysteine in pediatric patients with renal disease who do not have these complicating factors to determine the effects of renal disease on the levels of these two metabolites.

**Methods.** We measured plasma total homocysteine, S-adenosylhomocysteine, folate and vitamin B12 in 23 children between the ages of 1 and 17 who had a wide range of renal function.

**Results.** Plasma homocysteine had a slight inverse correlation with GFR:  $R^2=0.09$ . Plasma S-adenosylhomocysteine was strongly inversely correlated with GFR in a curvilinear fashion:  $R^2=0.76$ . None of the patients were folate or vitamin B12 deficient.

**Conclusions.** In children who have renal insufficiency without vascular disease there is a slight elevation of plasma homocysteine but plasma S-adenosylhomocysteine is highly correlated with glomerular filtration rate.

#### P134

##### EFFECTS OF "BEVITAMEL" SUPPLEMENTATION AND ERYTHROPOIETIN TREATMENT ON TOTAL PLASMA HOMOCYSTEINE LEVELS IN CHRONIC RENAL FAILURE PATIENTS

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A moderate elevation of tHcy levels is present in the early stage of chronic renal failure (CRF). It increases to parallel the degree of reduction in renal function, and persists after starting dialysis treatment. Total Hcy also might be influenced by various therapeutic protocols related to the primary kidney disease and/or to the renal function deterioration. The aim of the study was to evaluate the effects of "Bevitamel" (Sublingual tablet) - Westlake Laboratories, Inc. Cleveland Ohio, USA supplementation and the Erythropoietin (Epo) treatment separately on tHcy in stable predialysis CRF patients (pts). Totally 71 pts (43 m; 28 f) with predialysis CRF were evaluated twice in a period of two months each. In group A 26 of them were on conservative treatment of renal failure without "Bevitamel" or Epo; in group B - 24 pts on "Bevitamel" without Epo; and in group C - 21 pts only on Epo treatment. Total Hcy plasma levels (mcmol/L), Folate (nmol/L (Fol) and Vitamin B12 (pmol/L (B12) were determined using Bayer ACS: 180 Chemiluminescence's assays. The mean tHcy level did not change significantly during two months follow up period in pts in group A (28,9±11,9 to 28,6±14,29). In the "Bevitamel" group the mean tHcy decreased from 26,71±13,61 to 20,65±12,47 ( $p<0,1$ ). Plasma creatinine remained stable (204,94±107,44 and 210,18±128,63 respectively). In the group C haemoglobin increased from 103,78±13,63 to 118,35±10,34 g/L / $p<0,05$ /. No significant elevation was detected in the mean level of tHcy (31,75±11,43 and 36,35±11,42) and plasma creatinine (356,35±119,27 and 404,07±143,70 mcmol/L).

Various life style conditions and drugs, including Erythropoietin, have been reported to increase tHcy or resistance to VitB12 in chronic kidney disease pts (Korzets *et al.* 2000; Gallucci *et al.* 1999). There was no significant increase in the mean tHcy in our group of CRF pts successfully treated by Epo for two months. A promising trend was found in the tHcy in pts supplemented by vitamins combination - "Bevitamel".

#### P135

##### OXIDATIVE STRESS AND HOMOCYSTEINE IN UREMIC AND SIROLIMUS TREATED TRANSPLANTED PATIENTS

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**Background.** Total homocysteine (t-Hcy), Reactive Oxygen Species (ROS) and Total Antioxidant Capacity (TAC), markers of oxidative status, are involved in the development of accelerated atherosclerosis in patients with kidney disease (chronic renal failure or kidney transplantation). Aim To perform a cross-sectional study of oxidative status in nephropathic patients.

**Methods.** 22 kidney transplant patients (Tx) (plasma creatinine 1.2±0.2 mg/dL, aged 49±9 years), treated with sirolimus based immunosuppression, 19 patients with chronic renal failure (CRF) (plasma creatinine 3.5±1.2 mg/dL, aged 67±16 years) and 30 healthy subjects as controls (C) (aged 59±9 years) were evaluated. Hcy and ROS concentrations and TAC were measured by the relevant immunoenzymatic and spectrophotometric assays. Statistical analysis was performed by Student t-test, chi-square test and regression analysis.

**Results** are given as mean ± SD:

Analytes	ROS UCarr	TAC micromolHClO/mL	t-Hcy micromol/L
Reference interval	250-300	>350	<12
Tx (22)	388.4±75.3*°	431.6±81.1^§	15.4±05.6 **
CRF (19)	336.8±71.4°	362.4±24.5&	32.2±13.2 #
C (30)	287±59.8	383.4±24.1	8.3±3.5

\* $p<0.03$  vs CRF; ° $p=0.000$  vs C; ^ $p<0.0001$  vs CRF; § $p<0.003$  vs C; & $p<0.005$  vs C; \*\* $p<0.05$  vs C; # $p<0.0001$  vs C and Tx

ROS concentrations were higher in Tx than in CRF and in both patients' groups when compared to C. TAC levels were higher in Tx than in CRF and C. CRF had lower TAC than C. Hcy levels were higher in CRF and in Tx than in C and in CRF than in Tx: all these differences were statistically significant.

**Conclusions.** Different degrees of impaired kidney function seem to cause different conditions of hyperhomocysteinemia and oxidative stress. CRF patients show increased ROS production without any TAC increase. Normal renal function after transplantation seems to increase TAC, thus improving oxidative status, without, however, reducing ROS levels.

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**HOMOCYSTEINEMIA IN WOMEN AFFECTED BY STERILITY FROM SOUTHERN ITALY**D'Uva M,<sup>1</sup> Di Micco P,<sup>2</sup> Strina I,<sup>1</sup> Mollo A,<sup>1</sup> Lubrano Lobianco C,<sup>1</sup> Niglio A,<sup>2</sup> Barrella F,<sup>2</sup> De Placido G<sup>1</sup>*<sup>1</sup>Dipartimento di Scienze Ostetriche Ginecologiche e Medicina della Riproduzione, Area Funzionale di Medicina della Riproduzione ed Endoscopia Ginecologica, Università degli Studi di Napoli Federico II, Napoli; <sup>2</sup>IV Divisione di Medicina Interna, Seconda Università di Napoli, Napoli, Italy*

**Background.** Hyperhomocysteinemia has been frequently described as risk factor for unexplained recurrent pregnancy loss. Increased levels of homocysteine may be due to uncorrected food intake of folate and vitamin B12 (and/or B6), chronic diseases (kidney failure, diabetes, hypothyroidism) and inherited defects within the methionine-homocysteine pathway causing vitamin B12 and/or folate deficiency such as methylene-tetra-hydrofolate reductase (MTHFR) gene polymorphisms, in particular MTHFR C677T gene polymorphism. So, incorrect homocysteine metabolism may be related to gene and environmental factors. However, the association between hyperhomocysteinemia and sterility problems have been underlined only for recurrent pregnancy loss.

**Aims.** This study was undertaken to find out a possible relationship between sterility (primary sterility or secondary sterility due to recurrent pregnancy loss) and plasma homocysteine levels in a population of women from Southern Italy.

**Patients and Methods.** We selected 46 patients affected by primary or secondary sterility; 24 patients were affected by primary sterility, while 22 patients were affected by secondary sterility due to recurrent pregnancy loss. We already excluded patients affected by hydrosalpinx, uterine fibroids, luteal insufficiency, cytogenetical alterations, endocrinological diseases, immunological diseases. As control group we selected 32 age-matched women without sterility problems in their anamnesis and thrombotic disorders. A whole blood sample was collected in EDTA by venipuncture to test plasma homocysteine. Statistical analysis was performed by chi2 test; differences were considered to be significant if  $p < 0.05$ .

**Results.** The median fasting total plasma homocysteine concentration was  $19.7 \pm 4.5$   $\mu\text{mol/L}$  for the studied patients vs  $9.8 \pm 2.5$   $\mu\text{mol/L}$  of control group ( $p < 0.005$ ).

**Discussion.** These data raises lot of questions on the role of the homocysteine metabolism in sterility troubles. Although, increased homocysteine (i.e.  $>20$   $\mu\text{mol/L}$ ) and MTHFR C677T homozygosity have been already described as risk factors for deep venous thrombosis and recurrent pregnancy loss, few studies evaluated homocysteinemia in women affected by primary sterility. Further data, on large based populations, are needed to better understand not only the role of plasma homocysteine levels but also the role of the ethnic background (in particular MTHFR C677T and MTHFR A1298C) in these clinical settings in order to confirm our evaluation.

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**TOTAL PLASMA HOMOCYSTEINE AND FREE AMINO ACID VALUES IN PREGNANT WOMEN WITH FETAL NEURAL TUBE DEFECTS**Candito M,<sup>1</sup> Mengual R,<sup>1</sup> Boisson C,<sup>2</sup> Rudigoz R-C,<sup>2</sup> Luton D,<sup>3</sup> Journel H,<sup>4</sup> Oury J-F,<sup>5</sup> Roux F,<sup>5</sup> Gueant J-L,<sup>6</sup> Saura R,<sup>7</sup> Vernhet I,<sup>7</sup> Gaucherand P,<sup>2</sup> Muller F,<sup>5</sup> Bongain A,<sup>1</sup> Guidicelli B,<sup>5</sup> Heckenroth H,<sup>5</sup> Poulain P,<sup>8</sup> Blayau M,<sup>8</sup> Francannet C,<sup>9</sup> Roszyk L,<sup>9</sup> Brustia C,<sup>1</sup> Staccini P,<sup>1</sup> van Obberghen E<sup>1</sup>*<sup>1</sup>Nice, Hospital Pasteur and Archet; <sup>2</sup>Lyon, Hospital Croix Rousse and Edouard Heriot; <sup>3</sup>Paris, Hospital Robert Debré; <sup>4</sup>Vannes, <sup>5</sup>Marseille, Hospital Timone; <sup>6</sup>Nancy, Hospital Brabois; <sup>7</sup>Bordeaux, Hospital Pellegrin; <sup>8</sup>Rennes, Hospital Hôtel Dieu, <sup>9</sup>Clermont-Ferrand, Hospital Hôtel Dieu, France*

A decrease in dietary folate appeared as a neural tube defect (NTD) risk factor. Total plasma homocysteine (tHcy), inversely correlated with serum folate was observed increased in pregnant women with offspring affected by a NTD. Abnormalities in plasma free amino acids were also described in this pathology. In a prospective study in France, tHcy (by FPIA) and plasma free amino acids (amino acid analyzer Beckman) were assayed in 48 pregnant women (19 to 38 years) prior to elective abortion for NTD affected offspring and in 38 pregnant women (20 to 42 years) with a normal pregnancy. Normal range values, obtained in 102 healthy subjects (18 to 62 years) out of pregnancy were also given.

**Results.** tHcy mean value was not significantly increased in patients:  $6.56 \pm 2.66$   $\mu\text{mol/L}$ , compared with mean value in controls:  $6.06 \pm 1.38$ . Only one patient showed a hyperhomocysteinemia: 21.4  $\mu\text{mol/L}$ . Standard deviations were more important in patients than in the pregnant controls. Methionine mean value was not decreased (but increased in one patient), while there was an elevated mean serine value in patients, and no other major abnormalities was observed in amino acids involved in homocysteine metabolism. Some patients showed plasma amino acid values more important than in the control subjects out of pregnancy. It is the case of methionine (in one patient), citrulline (in another one) and arginine (in two patients). The increased values were not observed always in the same patients. In others two patients, a citrulline concentration decrease, with a little arginine concentration decrease and a moderate glutamate value increase, were observed; that have to be confirmed in larger studies. Periconceptional folic acid supplement is known to reduce the NTD frequency but the etiology of NTD is complex, with acquired and inborn metabolism defects, and the role of folic acid is not yet clearly elucidated.

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**MTHFR 677 GENOTYPE AND RISK OF NTD: DISTINGUISHING BETWEEN EMBRYONAL AND MATERNAL EFFECTS**Vermeulen HHM,<sup>1</sup> Straatman H,<sup>2</sup> van der Linden YJM,<sup>3</sup> Blom HJ,<sup>3</sup> den Heijer M<sup>1,2</sup>*<sup>1</sup>Department of Endocrinology, <sup>2</sup>Department of Epidemiology and Biostatistics, <sup>3</sup>Laboratory of Pediatrics and Neurology, Radboud University Nijmegen Medical Centre, The Netherlands*

The 677C>T variant in the gene coding for MTHFR, an enzyme in folate and homocysteine metabolism, is associated with the development of neural tube defects (NTD). The association could be the result of an embryonal genotype effect or a maternal genotype effect through the intrauterine environment. Further, the effect of the embry-

onal genotype could differ depending on whether the T-allele is of paternal or maternal origin (parent-of-origin effect). The aim of this study was to evaluate these effects by means of applying several tests and techniques. Data on 105 children with spina bifida, a type of NTD, and their parents were available. The 76 complete parent-child triads were analysed using the transmission disequilibrium test (TDT), the transmission asymmetry test (TAT), and a log-linear model with likelihood ratio tests. The TDT showed a preferential transmission of the T-allele to NTD children but was not significant (Chi-square 2.12;  $p=0.09$ ). The TAT pointed towards an influence of a maternally derived T-allele (Chi-square 3.32;  $p=0.07$ ). The log-linear model demonstrated an embryonal genotype effect and a significantly higher risk for a maternally versus a paternally derived T-allele. The relative risk (95% CI) estimates in the full log-linear model for the CT versus the CC child genotype were 0.8 (0.3-1.9) and 2.6 (1.1-6.0) for a paternal and maternal T-allele, respectively. The application of several methods of analysis showed that the relation between MTHFR 677 genotype and NTD seemed to be mediated through a maternally derived T-allele. However, the results were inconclusive and preliminary analysis of additional incomplete triads pointed towards the presence of a maternal effect through intrauterine environment.

### P139

#### HOMOCYSTEINE LEVELS IN NORMAL AND PREECLAMPTIC PREGNANCIES

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Endothelial dysfunction is claimed to be a leading cause of development of preeclampsia. The mechanism of the endothelial activation remains unclear for now. A number of predisposing factors are discussed. One of them is homocysteine as a known endothelial damage activator.

In our study 27 pregnant women were included: 15 with preeclampsia (group 1) and 12 healthy term pregnant women (group 2). They were admitted to the Maternal Risk Clinic between December 2003 and August 2004. Only singleton pregnancies without fetal malformations were included. The inclusion criteria were  $>140/90$  mmHg and proteinuria over 0.3 g/24h. Six of the women had a superimposed preeclampsia - 3 patients had diabetes, 2 - nephrolithiasis and 1 M. Basedowi. Two of the women were developed preeclampsia in a previous pregnancy.

The mean homocysteine level in the group1 was 11,01  $\mu$ mol/L and 6,24  $\mu$ mol/L, in the group2 ( $p<0.05$ ). Of all preeclamptic women only one (0.6%) had a serum homocysteine level lower than the mean for the group2. The women with a severe form had a significantly higher serum homocysteine levels than those with mild preeclampsia ( $p=0.025$ ). In 8 patients pregnancy was terminated before 34 weeks of gestation. Eight of the women delivered fetuses with low and extremely low birth weight.

In 6 of the women without accompanying disease a DNA analysis for inherited thrombophilia was made. One of them was homozygous for Factor V Leiden, one for C677T MTHFR and one was heterozygous for the same mutation of MTHFR. In 3 cases no mutations were estimated.

Our data show a significant difference between the mean levels of homocysteine in normotensive and preeclamptic patients and also between the mild and the severe forms of the disease. The results show a link between the serum

homocysteine levels and the development of preeclampsia. The results also support the hypothesis of a positive connection between the severity of the preeclampsia and the elevation degree of the homocysteine levels.

### P140

#### PLASMA HOMOCYSTEINE, VITAMINE VALUES AND ANTIEPILEPTIC THERAPY IN PREGNANCIES WITH NEURAL TUBE DEFECTS

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In pregnancy, a part folate deficiency, another acquired fetal neural tube defect (NTD) risk factor is antiepileptic therapy. Valproic acid (VA) has adverse effects: antifolate activity, alterations in the homocysteine cycle and a reduction in plasma vitamin B6. We report the cases of 4 women: MT, LC, NM, CA, with VA therapy, with a NTD affected offspring, even under 5 mg/day folic acid complement. Their results were compared with those of a woman, under VA: CW, and of 57 pregnant women, all with a normal pregnancy. Plasma homocysteine (Hcy), folate, vitamins B12, B6, and red cell folate levels were measured in samples. Some mutations were sought, involved in Hcy metabolism, linked with the folate metabolism: C677T (MTHFR gene), A2756G (MTR gene), A66G (MTRR gene), and codon 259 (TCII gene).

Results. the cases reported showed B6 decreased values, as another woman, CW, under VA, but with a normal pregnancy. However, under folic acid complement, plasma and erythrocyte folate values were lower in MT, CA, as in NM, than in CW and even than in LC. That might showed a trouble in the folate or in the homocysteine metabolisms, even if no hyperhomocysteinemia was seen (the subjects received folic acid supplement). In the cases, two heterozygote mutations were observed. CW, with a normal pregnancy, showed higher plasma and erythrocyte folate values than the cases and a sole heterozygote. Vitamin B6 deficiency decreased nicotinic acid synthesis from tryptophane, and so, decreased NAD<sup>+</sup> and NADP<sup>+</sup> synthesis, necessary to pyrimidic nucleotide synthesis. NTD are multifactorial diseases. In the cases, the association of a B6 vitamin decrease, due in particular to the therapy, with an unfavorable genetic profile, could be responsible of NTD. MT, with a periconceptional pyridoxine therapy, had a normal pregnancy, and a healthy child.

### P141

#### PREDICTORS AND EFFECTS OF FOLATE SUPPLEMENT USE: THE NORWEGIAN MOTHER AND CHILD COHORT STUDY

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Aims. The objective of this study was to investigate differences in prevalences of some life style factors and demographic parameters between folate supplement users and non-users in a large ongoing cohort study of Norwegian

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pregnant women, and to examine associations between selected pregnancy complications and outcomes and folate supplement use.

**Methods.** Supplement data of singleton births in 1999-2003 were linked to the Medical Birth Registry of Norway. Women taking folate daily (n=2,795, 12%) at least one month before pregnancy and three months after conception were compared to non-users and subjects who did not use folate regularly (n=20,780).

**Results.** Supplement users were more likely than non-users to be old ( $\geq 25$  years), married, to have low parity and to have planned their pregnancy. They more frequently reported *in vitro* fertilization and ovulation stimulation treatment and they smoked less than non-users. Premature births (<32 weeks), low birth weight (<2,500 grams) or placental abruption were not associated with folate use, but a reduced risk of preeclampsia was seen in subjects who reported periconceptional folate use (OR=0.69, 95% CI 0.52-0.93) after adjustment for marital status, maternal age, parity, infertility treatment and smoking. Exclusion of smokers did not alter this effect estimate.

**Conclusions.** For singleton births, our results show that folate supplement users and non-users were different on several characteristics relevant to pregnancy outcome. Users of folate had a reduced risk of preeclampsia.

### P142

#### THE EFFECT OF SUPPLEMENTS CONTAINING NATURAL FOLATE (MTHF) DURING PREGNANCY ON MATERNAL AND FOETAL PLASMA FOLATE AND tHcy

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**Background.** Pregnancy incurs an increased maternal folate requirement. If this is not met in the diet, folate deficiency ensues, causing anaemia and elevated plasma homocysteine (tHcy), with increased risk of complications. Accordingly, folic acid is widely prescribed during pregnancy. An alternative option for supplementation is the naturally occurring cofactor 5-methyltetrahydrofolate (MTHF). No studies have examined MTHF efficacy during pregnancy.

**Aims.** To determine whether supplementation with MTHF during late pregnancy would increase maternal folate status and lower plasma tHcy. A secondary objective was to determine whether concurrent intake of polyunsaturated fatty acids derived from fish oil might augment the tHcy lowering effect. **Methods:** Pregnant women were randomized to one of four supplementation groups: Placebo; MTHF (800µg 6R,S-MTHF); Fish oil (500 mg DHA plus 150 mg EPA); Fish oil plus MTHF. Bloods were collected at baseline (week 20 gestation), week 30 and delivery. Umbilical cord bloods were also collected. Plasma samples were analysed for folate and tHcy. The trial was performed at three clinical centres (in Spain, Germany and Hungary).

**Results.** Baseline plasma folate and tHcy were not significantly different by randomization group. Results at delivery are shown on Table 1. Supplementation with MTHF increased maternal plasma folate. No changes were observed in maternal tHcy or in neonatal folate or tHcy. There was no interaction between folate and PUFA.

**Conclusions.** Nutraceuticals containing MTHF would be beneficial during pregnancy to maintain a good maternal

folate status. Further research is required to evaluate the effect of folate containing supplements in controlling the level of homocysteine during this time.

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**Table 1. Maternal plasma folate and tHcy concentrations at delivery**

	Median Folate [IQR] µg/L	Median tHcy [IQR] µmol/L
Placebo (N=66)	5.45 [3.86-7.38]	7.09 [6.06-9.16]
MTHF (N=64)	11.94 [7.96-16.24]	7.09 [5.32-9.33]
Fish Oil (N=67)	4.76 [3.47-6.70]	7.36 [5.74-9.66]
MTHF+Fish Oil (N=60)	12.39 [9.34-15.97]	6.88 [5.22-9.69]
ANOVA	$p < 0.0001$	$p = 0.93$

### P143

#### VENOUS THROMBOEMBOLISM, CORONARY ARTERY DISEASE AND PLASMA HOMOCYSTEINE LEVELS IN FAMILIES WITH INHERITED THROMBOPHILIA

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**Background.** Pulmonary embolism (PE) is regarded as the leading cause of in-hospital morbidity and mortality. Inherited predisposition to venous thromboembolic disease (VTD) frequently remains clinically silent until additional environmental factors intervene.

**Aims.** To assess total plasma homocysteine (tHcy) levels in subjects from families whose probands had PE coexisting with factor V (FV) Leiden. **Methods.** In 18 members (9 M; 9 F) of three families (aged 6-58 years) the levels of plasma tHcy, folate and vitamin B12 were studied. Furthermore, all had been genotyped for identification of polymorphisms: FV Leiden, FII G20210A and methylenetetrahydrofolate reductase (677CT and 1298AC).

**Results.** Episodes of PE in 4 subjects (3 probands included), coronary artery disease in 4 subjects (1 proband included) and PA in 2 subjects (1 proband included) were found. Apart from 3 probands, FV Leiden was found in 10 other subjects (67% family members). Neither FII 20210GA, nor FII 20210AA genotypes were noted. General linear model analysis of vascular event (venous and arterial) based on FV Leiden as an independent variable, and fasting tHcy and age as co-variables, revealed a significant ( $R^2=0.56$ ;  $p=0.03$ ) correlation. Only mutated FV was significant in this model ( $p=0.044$ ), whereas the effects of age ( $p=0.07$ ) or tHcy ( $p=0.23$ ) remained non-significant. Also the calculated odds ratio (OR) for vascular event was non-significant, considering 12.0 µmol/L as the cut-off for tHcy level (OR 8.0; 95% CI 0.60-106.94). Lower folate (<4.0 ng/mL) in 4 and vitamin B12 (<200.0 pg/mL) levels in 2 subjects were reported.

**Conclusions.** Apart from FV Leiden, plasma tHcy levels may hardly be regarded as the sufficient predictor for development of venous and/or arterial thrombosis.

P144

**HOMOCYSTEINE AND RISK OF DVT IN PATIENTS WITH SPINAL CORD INJURY**

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Deep venous thrombosis (DVT) and pulmonary embolism are major causes of morbidity and mortality in patients with spinal injuries. A wide range of thromboprophylactic measures have been proposed but the optimum treatment and duration for thromboprophylaxis is unknown. No data are available on the role of thrombophilic risk factors. Aim of our study was to determine the thrombophilic risk profile of patients with spinal cord injury (SCI) with or without a previous deep venous thrombosis by evaluating: antithrombin, protein C and S, factor V Leiden, prothrombin polymorphism, homocysteine (Hcy), plasminogen activator inhibitor-1 (PAI.1) and lipoprotein(a) (Lp(a)). We studied 46 patients with SCI without DVT (group A) (35 M/11 F; age: 50.5 (21-82) and 43 patients with SCI and a previous episode of DVT (group B) (34 M/9 F; age: 50 (13-78). No patient had a deficiency of clotting inhibitors, and in only 1 patient of group A we found the presence of prothrombin polymorphism. Lp(a) levels were similar in the two groups (124 (1-487) mg/L vs 84.5 (1-1079) mg/L;  $p=ns$ ). Hcy and PAI-1 levels were significantly higher in group B with respect to A (Hcy: 12.3 (6.2-30.0) micromol/L vs 18.7 (5.4-35) micromol/L;  $p=0.003$ / PAI-1: 15.4 (1-40) IU/ml vs 10 (1-36.8) IU/mol;  $p=0.008$ ). At the multivariate analysis adjusted for age, sex, smoking, hypertension, dyslipidemia and BMI, independent risk factors for DVT in patients with SCI were hyperHcy (OR=3.6 (95% CI 1.4-9.5),  $p=0.007$ ) and high levels of PAI-1 (OR= 4.9 (1.7-14);  $p=0.002$ ). Our data demonstrate that hyperHcy and impaired fibrinolysis documented by high levels of PAI-1 are independent risk factors for the occurrence of DVT in patients with SCI, suggesting the possible benefit of a vitamin supplementation.

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**HYPERHOMOCYSTEINEMIA IS AN INDEPENDENT RISK FACTOR FOR THE OCCURRENCE OF DEEP VENOUS THROMBOSIS IN PATIENTS WITH SUPERFICIAL VEIN THROMBOSIS**

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Superficial vein thrombosis (SVT) is a common disease and the recent recognition of its association with deep venous thrombosis (DVT) and pulmonary embolism has revived the interest in this disease. Aim of our study was to determine the thrombophilic risk profile of patients with SVT by evaluating: factor V Leiden, prothrombin polymorphism, physiological clotting inhibitors, antiphospholipid antibodies, homocysteine, plasminogen activator inhibitor-1 (PAI-1) and lipoprotein (a). The study population consisted of 183 patients (138 F/ 45 M; age 42 (15-74) yrs) and 183 age and sex matched (138 F/ 45 M; age 42 (15-74) yrs) controls. In 103/183 (56.3%) patients the first episode of SVT occurred spontaneously, whereas in the others (43.7%) there was at least one circumstantial risk factor (34 during pregnancy; 10 after surgery; 22 on oral contraceptives/hormone replacement therapy; 14 after immobilization). At the multivariate analysis adjusted for age, sex, circumstantial and

thrombophilic risk factors, independent risk factors for SVT were: factor V Leiden (OR 12.3 (95% CI 5.0-30.2),  $p<0.000$ ); hyperhomocysteinemia (OR 6.2 (95% CI 2.8-13.7),  $p<0.000$ ); antiphospholipid antibodies (OR 5.1 (95% CI 1.6-16.4),  $p<0.006$ ) and prothrombin polymorphism (OR 3.5 (95% CI 1.1-12.5),  $p<0.000$ ). 106 patients (57.9%) had at least one recurrence of venous thromboembolism (either SVT or DVT), and 34/183 (13.1%) had a deep vein thrombosis after the first SVT. At the multivariate analysis, independent risk factor for a subsequent deep vein thrombosis were: prothrombin polymorphism (OR 12.6 (95% CI 2.9-53.9),  $p<0.001$ ) and hyperhomocysteinemia (OR 4.6 (95% CI 1.7-12.6),  $p<0.003$ ). Our findings indicate that a thrombophilic state is frequent in patients with SVT. In particular, we demonstrated that factor hyperhomocysteinemia and prothrombin polymorphism are independent risk factors for the occurrence of DVT after SVT. These alterations allow us to identify patients at higher risk of in whom antithrombotic prophylaxis is particularly warranted.

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**SEVERE HYPERHOMOCYSTEINEMIA RESULTING IN RECURRENT VENOUS THROMBOEMBOLISM, IN A PATIENT WITH MTHFR 677C>T HOMOZYGOSITY AND COMBINED VITAMIN B12 AND FOLATE DEFICIENCY**

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Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the vitamin B12 and folate dependent remethylation of methionine. Homozygosity for MTHFR 677C>T is linked to mild hyperhomocysteinemia (HHcy). Severe HHcy is found in classical homocystinuria (HCU) due to cystathionine beta-synthase (Cbs) deficiency which is clinically characterised by mental retardation, skeletal abnormalities, ectopia lentis and premature vascular disease. HHcy is now an established vascular risk factor. A total homocysteine (tHcy) rise of 1SD above the normal population mean is associated with a 60% rise in relative risk of cardiovascular disease (Boushey et al 1995). We report on a 40 year old male with recurrent venous thromboembolism referred for investigations for thrombophilia. He initially presented with a spontaneous right DVT and bilateral pulmonary emboli, managed with enoxaparin 40 mg/kg BD. Other problems include severe polyarticular gout, obesity with poor diet and alcoholism. There was no prior personal or family history of venous thromboembolism. Ophthalmology examination and his habitus were normal. Eight months later, after two days of excessive drinking and high protein intake, he represented with a further left pulmonary embolism while therapeutically anticoagulated on Enoxaparin. A Greenfield filter was inserted. Initial investigations: tHcy 235  $\mu$ mol/L (4-12). Serum folate 1 microg/L (2.7-20). Red cell folate was 45ug/L (150-1000) and Vitamin B12 of 100 ng/L (150-1000). A thrombophilia screen was normal including activated Protein C resistance and Prothrombin 20210G>A. He was homozygous for MTHFR 677C>T. Fibroblast studies for assessment of transsulfuration and remethylation revealed normal Cbs activity with normal methionine and serine formation ruling out severe forms of Cbs and MTHFR deficiency. At the time of the second thrombotic episode, tHcy was 351  $\mu$ mol/L and methionine 28  $\mu$ mol/L (6-40).

*Initial management.* Correction of the cofactor deficiencies

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was achieved by supplementation with vitamin B12 (IM) and folic acid, resulting in a fall of tHcy from 235 to 57  $\mu\text{mol/L}$ . Pyridoxine (B6) was commenced pending the result of CbS assay. At the second thrombotic episode, acute Hcy lowering treatment included stopping dietary protein and supplementing with a methionine free amino acid mixture. Betaine which remethylates homocysteine to methionine was started combined with all three cofactors and anticoagulation therapy, resulting in a tHcy level of 21  $\mu\text{mol/L}$  within 36 hours. Dietary protein was introduced gradually, titrated with tHcy levels. Betaine and amino acid mixture were eventually stopped and final protein intake restricted to 1g/kg/day pending investigations for HCU. Once stabilised, mean tHcy was 9.1  $\mu\text{mol/L}$  (range:5-21; n=12) and mean methionine 25.8  $\mu\text{mol/L}$  (range:12-39). A normal diet was introduced and B6 stopped when HCU was ruled out. He remains well with normal tHcy with regular control of serum B12 and folate levels.

**Conclusions.** This case demonstrates that MTHFR 677C>T homozygosity may cause severe HHcy and recurrent thromboembolisms in the presence of combined B12 and folate deficiency. Appropriate Hcy lowering therapy can result in a good outcome. It is important to maintain normal B12 and folate levels in patients with MTHFR 677C>T.

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#### MODERATE HYPERHOMOCYSTEINEMIA AND EARLY-ONSET CENTRAL RETINAL VEIN OCCLUSION

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Central retinal vein occlusion (CRVO) is the most common retinal vascular disease after diabetic retinopathy and affects mainly elderly patients, often with poor visual outcome and with a significant rate of recurrence. Both local and systemic risk factors have been found consistently associated with the onset of CRVO. Trombophilia defects are additional risk factors for CRVO; among these, the role of hyperhomocysteinemia (HHcy) is still debated, especially in patients with early-onset CRVO. There is scarce information on the role of post methionine-load HHcy in this clinical setting. We evaluated the prevalence of fasting and post methionine-load HHcy (delta PML, 8 hours), in a consecutive series of 58 patients with CRVO younger than 56 years (38 men and 20 women, mean age 40.3 yrs, 23 with the ischemic type of capillaropathy), 1 to 12 months after their last occlusive event, and in 103 controls (59 men and 44 women, mean age 39.6 yrs). Plasma folate, vitamin B12 and pyridoxal-5'-phosphate (PLP) levels were measured in 42 patients and 67 controls. Mantel-Haenszel odds ratios (adjusted for gender) in CRVO patients were 3.00 (95% confidence interval: 0.83-10.8) for fasting HHcy, 3.50 (1.07-11.4) for PML HHcy and 3.00 (1.18-7.6) for fasting HHcy and PML HHcy in subjects with normal fasting total homocysteine (tHcy) levels. Moderate HHcy was associated with reduced plasma levels of folate and PLP ( $p \leq 0.04$ ). There was no significant dependence of fasting and PML tHcy levels on any traditional risk factors evaluated, nor was the prevalence of HHcy different in subjects with the ischemic or edematous type of capillaropathy. These findings strongly suggest that moderate HHcy, either fasting or PML, is an independent risk factor for early-onset CRVO.

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