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ORIGINAL ARTICLE

Homocysteine induced cardiovascular events: a consequence of long term anabolic-androgenic steroid (AAS) abuse

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Objectives: The long term effects (>20 years) of anabolic-androgenic steroid (AAS) use on plasma concentrations of homocysteine (HCY), folate, testosterone, sex hormone binding globulin (SHBG), free androgen index, urea, creatinine, haematocrit (HCT), vitamin B12, and urinary testosterone/epitestosterone (T/E) ratio, were examined in a cohort of self-prescribing bodybuilders.

Methods: Subjects (n=40) were divided into four distinct groups: (1) AAS users still using AAS (SU; n=10); (2) AAS users abstinent from AAS administration for 3 months (SA; n=10); (3) non-drug using bodybuilding controls (BC; n=10); and (4) sedentary male controls (SC; n=10).

Results: HCY levels were significantly higher in SU compared with BC and SC ($p<0.01$), and with SA ($p<0.05$). Fat free mass was significantly higher in both groups of AAS users ($p<0.01$). Daily energy intake (kJ) and daily protein intake (g/day) were significantly higher in SU and SA ($p<0.05$) compared with BC and SC, but were unlikely to be responsible for the observed HCY increases. HCT concentrations were significantly higher in the SU group ($p<0.01$). A significant linear inverse relationship was observed in the SU group between SHBG and HCY ($r=-0.828$, $p<0.01$), indicating a possible influence of the sex hormones in determining HCY levels.

Conclusions: With mounting evidence linking AAS to adverse effects on some clotting factors, the significantly higher levels of HCY and HCT observed in the SU group suggest long term AAS users have increased risk of future thromboembolic events.

Sudden death and acute thrombotic events may represent under-appreciated risks of anabolic-androgenic steroid (AAS) use and therefore may be under-reported in the medical literature.¹ AAS are known to affect the haemostatic system,^{2,3} and systemic emboli and thrombotic complications have been reported in several androgen using athletes.^{4–6}

Recently, homocysteine (HCY), a by product of methionine metabolism, has been implicated in various cardiovascular related diseases. Several studies have established that the association between plasma HCY concentration and the risk of cardiovascular disease or severity of atherosclerosis is graded throughout the normal range from mild to elevated concentrations.^{7,8} HCY has been shown to dramatically impair vascular endothelial function through impairment of nitric oxide production potentiating oxidative stress and atherogenic development.^{9,10} Thus, one would suspect that elevated HCY levels may play a role in the development of systemic emboli and thrombotic complications in AAS users. However, few studies have looked at the effect of androgen use on HCY production. Zmuda *et al*¹¹ showed that short term administration of supraphysiological doses of testosterone enanthate (200 mg/week) did not affect fasting HCY levels in 14 weightlifters. In contrast, a more recent study identified acute hyperhomocysteinaemia in bodybuilders regularly self-administering supraphysiological doses of various AAS preparations.¹²

Possible mechanisms for accelerated cardiovascular disease with elevated HCY include endothelial cell injury,¹³ increased platelet adhesiveness,¹⁴ enhanced oxidation of LDL in the arterial cell wall,¹⁵ and through direct activation of the coagulation cascade.¹⁶

The hypothesis of the present investigation was that AAS use significantly elevated plasma HCY concentrations in a

cohort of long term (>20 years) AAS users. A secondary aim was to compare the findings with age matched control groups consisting of previous AAS using (but now abstinent) subjects versus resistance trained non-drug using subjects and sedentary controls. In addition to plasma concentrations of HCY, plasma folate, testosterone, sex hormone binding globulin (SHBG), free androgen index (FAI), urea, creatinine, haematocrit (HCT), and vitamin B12 (B12) were also measured. Deficiencies in plasma folate and B12 have been shown to lead to elevated HCY concentrations. Urinalysis for AAS was performed and the testosterone/epitestosterone (T/E) ratio was measured to confirm or refute the use of these substances. Finally, this study also examined the influence of sex hormones in determining HCY concentrations and outlined the possibility of long term AAS users developing future thromboembolic events.

METHODS

Ethical approval for the study was obtained from the university ethical committee and all subjects involved, having read experimental details, provided written consent. AAS using participants were recruited from a database of subjects who had been involved in previous studies,¹⁷ and from notices placed on bodybuilding web sites. Each AAS using recruit provided information stating that they had used AAS

Abbreviations: AAS, anabolic-androgenic steroid; B12, vitamin B12; BC, bodybuilding controls; FAI, free androgen index; FFM, fat free mass; FFMI, fat free mass index; HCT, haematocrit; HCY, homocysteine; ROS, reactive oxygen species; SA, AAS abstinent subjects; SC, sedentary controls; SD, standard deviation; SHBG, sex hormone binding globulin; SU, AAS using subjects; TBM, total body mass; T/E, testosterone/epitestosterone; tHCY, total HCY

Table 1 Anthropometric characteristics and drug history of subjects

	SU	SA	BC	SC
Age (years)	42.4±3.8	41.7±3.0	43.1±4.6	43.8±4.7
Height (m)	1.78±4.5	1.76±3.9	1.80±3.1	1.76±4.9
Body mass (kg)	109±13.2	107±7.7	91±7.4	84±13.4
BMI (kg/m ²)	34.4±2.7**	34.7±3.7**	28.3±2.0	27.0±2.6
Body fat (%)	14.0±3.4	14.7±3.8	14.7±1.4	18.7±5.6†
FFM (kg)	94±12.1**	91±11.3**	77±8.2	67±7.8
FFMI (kg/m ²)	29.6±2.9**	29.4±3.5**	23.8±2.3	21.6±3.6
Training (years)	25.4±2.1	22±3.5	18.5±2.3	0
AAS use (years)	21±2.1	20.7±2.8	0	0

Values are means ± SD. AAS, anabolic-androgenic steroids; BC, bodybuilding controls; BMI, body mass index; FFM, fat free mass; FFMI, fat free mass index; SA, AAS abstinent subjects; SC, sedentary controls; SU, AAS using subjects.

*p<0.05, significantly greater than BC; **p<0.01, significantly greater than SC; †p<0.05, significantly greater than SU, SA, and BC.

in various doses in cyclical fashion over the previous 20 years (table 1).

The sample comprised of past and present, amateur, national, and international bodybuilding competitors. Only 10 of the 30 contacted subjects using AAS agreed to participate in this study because of the inherent underground and secretive nature of the activity. Subjects were divided into four distinct groups: AAS users who were still using AAS at the time of testing (SU; n = 10), AAS users who had been abstinent for more than 3 months (SA; n = 10), bodybuilding controls who did not use any pharmacological ergogenic aids (BC; n = 10), and sedentary male controls (SC; n = 10). The SA group had abstained from AAS use for a minimum of 12 weeks prior to examination. The power of the test was calculated to determine a confidence interval of 95%, p<0.05, for 10 subjects in each group. Urinalysis was performed in the IOC accredited laboratory, Drug Control Centre, Kings College London, UK, to provide confirmation of AAS use in all groups and to eliminate potential positive samples. Subjects presenting with positive urine samples were included solely in the SU cohort. T/E ratios in the SU group greatly exceeded legal levels for athletic competition. AAS metabolites for the SU group are outlined in table 2, demonstrating polypharmacy.

Each subject recorded the quantity and type of food and beverages ingested for a randomly assigned 3 day period. This information was analysed for protein concentrations to determine if creatinine levels had been affected by each subject's protein intake (CompEat, Grantham, UK). Total body mass (TBM) was measured using a balanced weighing scales (Seca, Cardiokinetics, Salford, UK) and height using a stadiometer (Seca). Body mass index (BMI) was calculated by dividing subject weight in kilograms by the square of the subject's height in metres. Body fat percentage was determined using hydrostatic weighing. Following a familiarisation trial, underwater weight was determined five times. The mean of the five trials was used as the criterion value. Body

density was determined using the equations of Siri,¹⁸ and percentage fat values calculated using the equation of Durnin and Womersley.¹⁹ Fat free mass (FFM) was calculated by subtracting fat mass from TBM. Fat free mass index (FFMI) was calculated by dividing the subject's FFM in kilograms by the square of the subject's height in metres.

Venous blood was sampled using the standard venepuncture method, from an antecubital vein following an overnight fast and 30 min supine rest.²⁰ Morning blood samples were taken (between the hours of 10.00 a.m. and 11.00 a.m.) to minimise the effect of day time biological variation in male sex hormone concentrations.²¹ Blood samples were appropriately sampled, centrifuged, and immediately stored at -70°C until analysis. Testosterone was analysed on a Bayer Advia Centaur immunoassay analyser, employing chemiluminescence detection (Bayer Diagnostics, Newbury, Berks, UK). The intra- and inter-assay coefficients of variation for testosterone were each 7.4%. SHBG and FAI were determined by enzyme immunoassay (IDS, Boldon, Tyne & Wear, UK). The intra- and inter-assay coefficients of variation for SHBG were 0.6% and 1.6%, respectively.

Urea and creatinine were analysed using dry chemistry slide technology on an Ortho Vitros 950 analyser (Ortho Clinical Diagnostics, Amersham, Bucks, UK). The intra- and inter-assay coefficients of variation for creatinine were each 1.8%.

Haematology analyses were performed using a Beckman Coulter GEN-S (Beckman Coulter, High Wycombe, Bucks, UK). Total HCY (tHCY) was measured from plasma blood samples by fluorescence polarisation immunoassay using the IMx system analyser (Abbott Laboratories, Maidenhead, UK). The intra- and inter-assay coefficients of variation for HCY were each 3.6%.

B12 and folate were analysed using microparticle enzyme immunoassay technology using the IMx system (Abbott Laboratories). The coefficients of variation for B12 and folate were each 3.3%.

Table 2 Various preparations used by the SU group at the time of testing, as identified in training diaries

Steroid	No. of users (n = 10)
Deca Durabolin (nandrolone decanoate)	5
Testosterone	6
Dianabol (methandienone)	4
Anadrol (oxymetholone)	2
Winstrol/Stromba (stanozolol)	3
Primobol (methenolone enanthate)	5
Equipoise (boldenone)	2
Finajet (trenbolone)	1

Statistical analysis

Data were analysed using the SPSS 10.0 for Windows statistical package. Group differences were analysed using a one way ANOVA followed by a post-hoc Tukey test. The Pearson product bivariate procedure was used to examine correlations between variables. Statistical significance was accepted at the p<0.05 level. The data are presented as means ± standard deviation (SD).

RESULTS

Values for BMI, FFM, and FFMI were not different between SU and SA, but were significantly higher in both AAS using groups than in BC and SC (p<0.01). The SC group had a

Table 3 Male serum sex hormone data for subjects at the time of testing

	SU	SA	BC	SC	Normal range
Testosterone (nmol/l)	69.4±7.1*	17.1±5.9	16.6±4.9	14.0±3.4	8–30
SHBG (nmol/l)	3.4±2.3**	14.4±6.4†	23.7±8.9	28.0±12.4	15–100
FAI (%)	40.4±28.8***	1.6±1.5	0.7±0.3	0.56±0.2	14–128

Values are means ± SD. AAS, anabolic-androgenic steroids; BC, bodybuilding controls; FAI, free androgen index; SA, AAS abstinent subjects; SC, sedentary controls; SHBG, sex hormone binding globulin; SU, AAS using subjects.

* $p < 0.05$, significantly different compared to SA; ** $p < 0.01$, significantly different compared to BC; *** $p < 0.001$, significantly different compared to SC; † $p < 0.05$, significantly lower than BC and SC.

significantly higher percentage of body fat than the bodybuilding groups (table 2).

Testosterone was significantly higher in SU ($p < 0.05$) compared with SA, BC, and SC. SHBG was significantly lower in SU ($p < 0.01$) compared with the three other groups. SHBG was also significantly lower in SA ($p < 0.05$) compared with BC and SC. FAI was significantly higher in the SU group compared with the three other groups ($p < 0.001$) (table 3).

HCY was significantly higher in SU ($p < 0.01$) and SA ($p < 0.05$) compared with BC and SC. HCT was significantly higher in SU compared with BC and SC ($p < 0.01$).

Creatinine was significantly lower in SC compared with SU ($p < 0.01$) and with SA ($p < 0.05$), but not with BC; SU and SA were just outside the reference range. Levels of B12, folate, and urea were not significantly different between groups. The normal urea indicated that subjects were euhydrated (table 4).

Daily energy intake (kJ) and daily protein intake (g/day) were significantly higher ($p < 0.05$) in SU and SA compared with BC and SC.

Pearson product correlation analyses were subsequently performed to further assess similarities and differences in plasma and serum chemistry profiles, first by pooling the subject groups and then by examining individual group correlations.

HCY and SHBG demonstrated a significant inverse relationship in both AAS using groups but not with controls (SU: $r = -0.828$, $p < 0.01$; SA: $r = 0.602$, $p < 0.05$). A significant relationship was also demonstrated between HCY and creatinine solely in SU ($r = 0.602$, $p < 0.05$). Correlation analyses also revealed a significant relationship between creatinine and FFM when combining the groups ($r = 0.708$, $p < 0.01$), and when assessed individually for SU ($r = 0.664$, $p < 0.05$), SA ($r = 0.710$, $p < 0.01$), and SC ($r = 0.743$, $p < 0.05$), but not for BC.

DISCUSSION

The elevated HCY levels observed in the SU group is consistent with recent work¹² but does not agree with the lack of HCY alteration reported in males treated with supraphysiological doses of testosterone.¹¹ Differences may

be due to the fact that Zmuda and colleagues administered therapeutic doses of testosterone enanthate for 3 weeks. Such a relatively brief intervention may not be sufficient to elicit augmentation of HCY. In addition, the particular drug used by Zmuda and co-workers (testosterone enanthate) may not affect HCY levels to the same extent as the multi-drug regimen, including ingestion of oral 17- α -allylated AAS, used in this study and that used in the study of Ebenbichler *et al.*¹² Dietary differences may have also existed between the bodybuilding and weightlifting groups in these studies. The significantly higher levels of SHBG in the SA group compared with both BC and SC indicates prolonged suppression of the globulin fraction following AAS withdrawal. Further indirect confirmation of AAS use may be provided by the greater FFM observed in both AAS using groups which are vastly in excess of the values (FFMI > 25 kg/m²) proposed by Kouri *et al.*²² The greater FFM values observed in both groups of AAS using bodybuilders is consistent with the assertion that AAS use in combination with resistance training significantly increases lean body mass,²³ which may be maintained for prolonged periods following AAS withdrawal as shown in postmenopausal females.^{24, 25}

The lack of discernable differences between groups in levels of folate or B12 indicates that HCY differences were not caused by dietary deficiencies in these nutrients. Dietary protein intake was significantly higher amongst the AAS using bodybuilders, mainly through the consumption of supplementary high-protein beverages. Although HCY levels were higher in the SU group, there was no difference in dietary protein intake between both AAS using groups, which suggests that diet alone is unlikely to be responsible for the higher HCY concentrations. Investigation of vitamin B6 concentrations may have been interesting, but the specific assay was unobtainable. Vitamin B6 concentrations would have provided complementary data only, and for the purposes of this study would not have affected outcomes. In humans, the chronic effects of increased dietary methionine have been investigated, whereby methionine supplementation (25 mg/kg/day) for 14 days did not seem to affect the results of a methionine loading test or fasting concentrations of either methionine or HCY.²⁶ The acute effects of a

Table 4 Test results, energy intake, and daily dietary protein intake for AAS using and control groups

	SU	SA	BC	SC
HCY (μ mol/l)	13.2±2.9**	10.4±1.6*	8.7±1.4	8.6±2.1
B12 (pmol/l)	272±176	410±203	428±145	290±169
Folate (ng/ml)	7.1±3.1	6.5±2.1	6.5±2.7	9.7±3.4
Urea (mmol/l)	5.9±2.9	5.9±0.9	4.5±1.7	4.2±1.5
Creatinine (mmol/l)	107±14.5	102±17.2	96.1±9.2	83.3±13.6††
HCT (%)	55.7±2.1**	48.2±2.8	45.6±2.2	43.1±2.6
Dietary information (n = 10)				
Energy intake (kJ/day)	18 400±4130*	17 100±2120*	14 900±1725	13 440±2140
Protein intake (g/day)	215±70*	190±55*	130±25	115±20

Values are means ± SD. BC, bodybuilding controls; SA, AAS abstinent subjects; SC, sedentary controls; SU, AAS using subjects.

* $p < 0.05$, significantly greater than BC; ** $p < 0.01$, significantly greater than SC; † $p < 0.01$, significantly lower than SU; †† $p < 0.05$, significantly lower than SA.

What is already known on this topic

- AAS are known to cause marked dyslipidaemia and there are individual case reports of sudden death following AAS use
- Mildly increased levels of homocysteine are associated with venous thrombosis and endothelial dysfunction
- Homocysteine levels have been shown to be elevated by AAS use

protein-rich diet involving supplemental protein drinks on plasma HCY levels warrants further and more careful evaluation.

Serum creatinine, regarded as an indicator of renal function, has been shown to be a strong determinant of plasma HCY concentration.^{27, 28} In the present study, a weak overall relationship was observed between creatinine and HCY, and was only significant in the SU group ($r = 0.602$, $p < 0.05$). Creatinine concentration was significantly lower in the control group compared with the AAS using body-builders, and Pearson product correlation revealed a significant relationship between creatinine and FFM ($r = 0.708$, $p < 0.01$). Given that creatinine is one of the most specific indices of total body skeletal muscle mass²⁹ and a marker with low sensitivity for early decline in renal function, the higher creatinine levels exhibited by the AAS using groups are likely to be a function of muscle mass rather than any renal impairment resulting from AAS consumption.

Recent clinical and epidemiological studies suggest a role of HCY in vascular disease; tHCY is associated with intima-media thickness in the carotid artery suggesting that this analyte may be a marker of early carotid atherosclerosis.³⁰ The precise mechanism linking it to cardiovascular disease is unknown, but it has been proposed that HCY induces endothelial dysfunction and atherosclerosis by generation of reactive oxygen species (ROS). Perez-de-Arce *et al* found that HCY induced ROS (1.85-fold) and that expression of the mitochondrial biogenesis factors, nuclear respiratory factor-1 and mitochondrial transcription factor A, were significantly elevated in HCY treated cells.³¹ These changes were accompanied by an increase in mitochondrial mass and higher mRNA and protein expression of the subunit III of cytochrome *c* oxidase. These effects were significantly prevented by pre-treatment with the antioxidants catechin and trolox, which modulated the adverse vascular effects of HCY.³¹ This may be one of the mechanisms whereby HCY can induce vascular events. AAS have been shown to produce marked dyslipidaemia,³² and the decrease in high density lipoprotein cholesterol and elevation of the low density lipoprotein cholesterol:high density lipoprotein cholesterol ratio which is a known effect of oral ingestion of 17- α -allylated AAS, associated with the very significant testosterone levels in SU compared with the other three groups, may be another mechanism which increases the susceptibility of the SU cohort to cardiovascular events. The AAS also increase platelet aggregability^{33, 34} and adversely alter endothelial function.³⁵ Considering the lack of classic risk factors (obesity, smoking, hypertension, inactivity), the youth of the subjects, and the lack of atherosclerotic lesions in a number of AAS related cardiovascular events, it is not unreasonable to suggest atherothrombotic rather than atherogenic mechanisms for sudden death as proposed by Ferenchick and colleagues.³³ In the absence of direct evidence, the ever-emerging case reports of thromboembolism^{2, 6, 36-38} in AAS users provides corroborating support for possible prothrombotic effects of supraphysiological AAS administration.

What this study adds

- Homocysteine levels may be an easily identifiable and powerful independent risk factor for cardiovascular disease
- The three AAS abusers who died during this study had elevated homocysteine levels greater than the SU group as a whole
- The mechanism of death could be related to the production of reactive oxygen species caused by the elevated homocysteine, which may be attenuated by the use of antioxidants

In conclusion, the findings from this study suggest that the long term use of supraphysiological doses of AAS is associated with hyperhomocysteinaemia and dramatically elevated HCT concentrations.

Previous research has indicated that when HCY concentrations are less than 9 $\mu\text{mol/l}$, there is a 3.8% increase in mortality measured over 4.6 years. When these concentrations exceed 15 $\mu\text{mol/l}$, there is a 24.7% increase in mortality measured over the same time period. In an analysis in which patients with HCY levels below 9 $\mu\text{mol/l}$ were used as the reference group, from 9–14.9 $\mu\text{mol/l}$ the mortality ratio was 1.9, from 15–19.9 $\mu\text{mol/l}$ it was 2.8, and greater than 20 $\mu\text{mol/l}$ it was 4.5.¹⁰

Nygard's work has shown that for every 5 $\mu\text{mol/l}$ increase in tHCY, the odds ratio for an increase in risk of coronary artery disease (CAD) was 1.6 (95% confidence interval (CI), 1.4 to 1.7) for men and 1.8 (95% CI, 1.3 to 1.9) for women. A total of 10% of the general population's CAD risk appears attributable to tHCY. The odds ratio for cerebrovascular disease (5 $\mu\text{mol/l}$ tHCY increment) is 1.5 (95% CI, 1.3 to 1.9). A 5 $\mu\text{mol/l}$ reduction in plasma HCY will prevent 10% of CAD deaths in men. A 5 $\mu\text{mol/l}$ tHCY increment elevates CAD risk to the same extent as a cholesterol increase of 0.5 $\mu\text{mol/l}$.³⁹

Additionally there have been an increasing number of case reports of sudden death following AAS use and even mild elevations of HCY are associated with venous thrombosis. In the present study, three individual subjects died suddenly. Their mean age ($n = 3$) was 43 years. The mean HCY value of the SU group ($n = 10$) was $13.2 \pm 2.9 \mu\text{mol/l}$. The HCY levels of the deceased were 15, 16, and 18 $\mu\text{mol/l}$. Post mortem examination identified cardiovascular disease as a cause of death in each case. The elevated HCY and associated HCT levels which appear to be a consequence of AAS use cannot be ruled out as causative in any of these cases. The findings of this study suggest that AAS are detrimental to cardiovascular health and appear to be implicated in cardiovascular mortality in long term AAS abuse.

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Competing interests: none declared.

Ethical approval for this study was obtained from the university ethical committee and all subjects involved, having read experimental details, provided written consent. AAS using participants were recruited from a database of subjects who had been involved in previous studies.

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