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## **Coagulation activity before and after acute psychosocial stress increases with age**

**Running title:** Age, coagulation activity and stress

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## Abstract

**Background:** It is known that hypercoagulation occurs in response to acute stress and that a shift in hemostasis towards a hypercoagulability state occurs with age. However, it is not yet known whether acute stress further increases hypercoagulation in older individuals, and thus may increase their risk for CVD.

**Objective:** To assess whether stress further increases hypercoagulation in older individuals, we investigated whether acute stress-induced changes in coagulation parameters differ with age.

**Methods:** 63 medication-free non-smoking men aged between 20 to 65 years (mean±SEM:36.7±1.7) underwent an acute standardized psychosocial stress task combining public speaking and mental arithmetic in front of an audience. We measured plasma clotting-factor-VII activity (FVII:C), fibrinogen, and D-dimer at rest, immediately, and 20 min after stress.

**Results:** Increased age predicted greater increases in fibrinogen ( $\beta=.26, p=.034; \Delta R^2=.05$ ), FVII:C ( $\beta=.39, p=.006; \Delta R^2=.11$ ), and D-dimer ( $\beta=.46, p<.001; \Delta R^2=.15$ ) from rest to 20 min after stress independent of body mass index (BMI) and mean arterial blood pressure (MAP). General linear models revealed significant effects of age and stress on fibrinogen, FVII:C, and D-dimer (main effects:  $p's < .04$ ), and greater D-dimer stress reactivity with older age (interaction age-by-stress:  $F(1.5/90.4)=4.36, p=0.024, f=.33$ ).

**Conclusions:** Our results suggest that acute stress might increase vulnerability in the elderly for hypercoagulability and subsequent hemostasis-associated diseases like CVD.

**Keywords:** Age, coagulation, clotting factor VII:C, D-dimer, fibrinogen, psychological stress

## **Abbreviations**

CVD = Cardiovascular Disease

ACS = Acute Coronary Syndromes

BMI = Body Mass Index

MAP = Mean Arterial Blood Pressure

BP = Blood Pressure

SEM = Standard Error of Means

AUC = Area Under The Curve

TSST = Trier Social Stress Test

FVII:C = Clotting Factor VII Activity

FVIII:C = Clotting Factor VIII Activity

PAI = Plasminogen Activator Inhibitor

TAT = Thrombin-Antithrombin

vWF = von Willebrand Factor

## 1. Introduction

The incidence of thrombotic cardiovascular disease increases with age (1, 2). In order to explain the higher thrombotic risk and ultimately cardiovascular mortality with increasing age, several studies have attempted to elucidate changes in hemostasis during aging (3, 4). Plasma concentrations of certain coagulation factors including fibrinogen and factor VII as well as the coagulation activation marker D-dimer increase in healthy persons parallel with age. Fibrinogen, a primary risk factor for thrombotic disorders (5, 6), increases in increments of 10 mg/dL per each decade (3). Fibrinogen plays a role in the development of atherosclerotic plaques by migrating into the intima of injured vessel walls, where it forms cross-linked fibrin, mural thrombi, and fibrin degradation products (7). This may lead to the augmentation of inflammatory mediators that promote cell migration and adhesion, platelet aggregation, and foam cell lipid uptake (8). Similarly, factor VII (FVII), which has been identified as an independent risk factor for cardiovascular events (9), also progressively increases with age (10, 11). Although there is less conclusive information on the atherothrombotic role of FVII:C (12), it plays a pivotal role in initiating the extrinsic coagulation pathway, and therefore may play a pathophysiologic role in atherothrombosis. The fibrin degradation fragment D-dimer also positively correlates with age (13). Fibrin D-dimer is a valid biochemical marker of both fibrin formation following activation of the coagulation cascade, and fibrin degradation by the fibrinolytic system (14, 15), while fibrinogen and FVII:C are simply markers of coagulation activation resulting in fibrin formation downstream in the coagulation cascade. After the fibrin plug is created, the fibrinolytic system degrades it to produce degradation products, such as D-dimer (16). Age-associated increases in D-dimer appear to result from both increased coagulation, as well as, decreased fibrinolytic activity as evidenced by higher plasminogen activator inhibitor (PAI)-1 levels (3, 17).

Accumulating evidence suggests a strong impact of mental stress on the pathogenesis of cardiovascular disease and acute coronary syndromes (ACS) (18, 19). A recent review suggests that psychological stress may act as a potential trigger for the onset of ACS via effects on hemostasis (20). Patients whose ACS had been preceded by acute emotion triggering stress show heightened platelet activation in response to psychological stress compared to patients without emotion triggering ACS (21). In healthy individuals acute mental stress activates both the coagulation and the fibrinolysis components of hemostasis to result in net hypercoagulability (20, 22). Acute psychological stress-induced increases have been observed in several hemostatic parameters such as FVII:C, FVIII:C and FXII:C, thrombin-antithrombin (TAT) complex, fibrinogen, von Willebrand Factor (vWF), and D-dimer levels (20).

Given that age is a cardiovascular thrombotic risk factor mediating this risk at least in part via elevated coagulability, the influence of age on mental stress-induced changes in hemostasis has received surprisingly little attention. Studies from our own group and from other investigators assessed the effects of acute stress on coagulation parameters in different age groups of healthy subjects including younger (23), middle-aged (24-27), or elderly persons (28). For instance, in a naturalistic setting of chronic psychosocial stress, highly stressed dementia caregivers, showed a linear increase in resting D-dimer levels along with older age, whereas D-dimer did not correlate with age in a non-caregiving control group (29). However, none of these studies assessed a broader age range for associations between age and coagulation reactivity to acute stress. We therefore aimed to elucidate whether stress-induced changes in coagulation parameters differ with age in a group of medication-free non-smoking men aged between 20 to 65 years. In light of previous research findings, we measured the coagulation parameters

fibrinogen and FVII:C, as well as the hypercoagulation marker D-dimer immediately before as well as several times after an acute psychosocial stress task.

## **2. Methods**

### **2.1 Study participants**

The Ethics Committee of the State of Zurich, Switzerland, formally approved the research protocol. The final study sample consisted of 63 subjects who provided written informed consent. The study was conducted between April 2004 and August 2005. We intentionally recruited non-smoking men of a wide age range between 20 and 65 years with an optimum of at least one person per each 2 years of age who were in excellent physical and mental health confirmed by an extensive health questionnaire (30) and telephone interview. Specific exclusion criteria, obtained by subjects' self-report, were: regular strenuous exercise, alcohol and illicit drug abuse; any heart disease, varicosis or thrombotic diseases, elevated blood sugar and diabetes, elevated cholesterol, liver and renal diseases, chronic obstructive pulmonary disease, allergies and atopic diathesis, rheumatic diseases, and current infectious diseases. In addition, participants were included only if they reported taking no prescribed and/or over the counter medication, either regularly or occasionally and if their blood pressure was in the normotensive or moderately hypertensive range (systolic BP < 160 mmHg and diastolic BP < 100 mmHg). If the personal or medication history was not conclusive, the subjects' primary care physician was contacted for verification.



## **2.2 Stress protocol**

Subjects were tested between 2:00 pm and 4:00 pm. They had abstained from physical exercise, alcohol, and caffeinated beverages since the previous evening. We used the Trier Social Stress Test (TSST) combining a 5-min preparation phase followed by a 5-min mock job-interview, and 5-min mental arithmetic in front of an audience (31). The TSST evokes reliable physiological responses in different biological systems, including coagulation factors investigated in the present study (25). During recovery, subjects remained seated in a quiet room for 40 min.

Blood pressure was measured immediately before and 40 min after stress by sphygmomanometry (Omron 773, Omron Healthcare Europe B.V. Hoofddorp, The Netherlands) and mean arterial blood pressure (MAP) was calculated by the formula ( $2/3 * \text{mean diastolic BP} + 1/3 \text{ mean systolic BP}$ ).

Blood for coagulation measures was obtained immediately before stress, immediately after stress, and 20 min after stress. Samples of stimulated whole saliva (by chewing on cotton rolls) were taken immediately before the TSST, as well as 0, 10, 20, 30, 40, 50 and 60 minutes after completion of the TSST for determination of salivary free cortisol levels.

## **2.3 Biochemical analyses**

Venous blood was drawn through an indwelling forearm catheter into polypropylene tubes containing 3.8% sodium citrate and centrifuged at 2,000 X g for 20 min at 4° C. Obtained plasma was immediately aliquoted in polypropylene tubes and frozen at -80°C. All analyses of coagulation factors used the BCS® Coagulation Analyser (Dade Behring, Liederbach,

Germany). Determination of FVII:C used standard coagulometric methods using factor-deficient standard human plasma and reagents (Dade Behring) and plasma fibrinogen was determined using a modified Clauss method (Multifibren U, Dade Behring). Plasma D-dimer was measured by means of an enzyme-linked immunosorbent assay (Asserachrom Stago, Asnières, France). Inter- and intra-assay coefficients of variation (CV) were <10% for all coagulation measures.

For cortisol, saliva was collected using Salivette collection devices (Sarstedt, Rommelsdorf, Germany), which were stored at -20°C until biochemical analysis. Cortisol concentrations were determined using a commercially available competitive chemiluminescence immunoassay with high sensitivity of 0.16 ng/ml (LIA, IBL Hamburg, Germany). Intra- and inter-assay variability was less than 7.7 and 11.5%, respectively.

## **2.4 Statistical analyses**

Data were analyzed using SPSS (version 13.0) statistical software package (SPSS Inc., Chicago IL, USA). All tests were two-tailed with level of significance set at  $p \leq .05$ . Using the trapezoid formula, we calculated areas under the total response curves, expressed as area under the measured time-points with respect to ground (AUC<sub>g</sub>) for all coagulation measures and cortisol (32). Prior to statistical analyses all data were tested for normality using the Kolmogorov-Smirnov test. Coagulation values and coagulation AUCs were logarithmically transformed to achieve normal distributions. For reasons of clarity, we provide untransformed data. For assessment of associations between age and coagulation activity before and after stress, we first calculated linear regression analyses with the respective coagulation measure as the dependent variables and age as independent variable. All independent variables were simultaneously forced into the regression equations. We used coagulation baseline measures as

the dependent variables to assess associations between age and coagulation activity at rest. We employed AUC measures of the coagulation parameters to assess associations between age and stress-induced coagulation changes. In light of previously reported associations between BMI and MAP with coagulation parameters at rest and in response to stress (33, 34), we controlled for BMI and MAP. We entered these parameters as predictors in all analyses. In an additional set of analyses, we also considered cortisol measures since resting cortisol levels and cortisol reactivity have also been shown to affect coagulation activity (34, 35). Relation of morning serum cortisol to prothrombotic activity in women with stable coronary artery disease. *J Thromb Thrombolysis* (in press).

Following our previous observations (34), the optimal total sample size to predict stress reactivity in coagulation parameters was  $n=68$  for detecting a conservatively expected medium effect size of 0.15 in regression analyses with a power of 0.80 using 2 predictors.

Second, we further tested regression results by performing general linear models with repeated measurement for each coagulation parameter as dependent variable and with age as continuous independent variable. In these analyses, we again controlled for BMI and MAP. We performed Huynh-Feldt correction for repeated measures. For illustrative purposes, we categorized the study group based on their age into three groups of subjects with younger (20-29yrs,  $n=30$ ), middle (30-45 yrs,  $n=17$ ) and older age (46-65 yrs,  $n=18$ ).

### **3. Results**

#### **3.1 Subjects' characteristics**

Table 1 presents the characteristics of the 63 men studied. The mean age of our study participants was 37 years, the mean BMI was 24.8, and mean MAP was 94.7 mmHg.

### 3.2 Regression analyses

#### *Age and coagulation activity at rest*

*D-dimer at rest:* Higher age significantly predicted D-dimer levels at rest ( $\beta=.46$ ,  $p<.001$ ;  $\Delta R^2=.15$ ) independent of MAP ( $p=.11$ ) and BMI ( $\beta=.50$ ,  $p<.001$ ;  $\Delta R^2=.18$ ) with the total model explaining 55 % of the total variance in D-dimer resting levels ( $F(3/59)=24.24$ ,  $p<.001$ ).

*FVII:C at rest:* Higher age significantly predicted FVII:C at rest ( $\beta=.39$ ,  $p=.006$ ;  $\Delta R^2=.11$ ) independent of MAP ( $p=.61$ ) and BMI ( $p=.18$ ). The total model explained 22% of the total variance in FVII:C ( $F(3/59)=5.53$ ,  $p=.002$ ).

*Fibrinogen at rest:* Higher fibrinogen at rest was significantly predicted by higher age ( $\beta=.26$ ,  $p=.034$ ;  $\Delta R^2=.05$ ) independent of MAP ( $p=.99$ ) and BMI ( $\beta=.47$ ,  $p<.001$ ;  $\Delta R^2=.16$ ) with the total model explaining 40 % of total variance in fibrinogen resting levels ( $F(3/59)=13.14$ ,  $p<.001$ ).

#### *Age and coagulation change between rest and 20 min after stress*

*D-dimer area under the curve:* Higher age predicted higher D-dimer AUC ( $\beta=.51$ ,  $p<.001$ ;  $\Delta R^2=.18$ ) independent of MAP ( $\beta=-.20$ ,  $p=.063$ ) and BMI ( $\beta=.46$ ,  $p<.001$ ;  $\Delta R^2=.15$ ). The whole model explained 55% of the observed variance in D-dimer AUC ( $F(3/59)=23.69$ ,  $p<.001$ ).

*FVII:C area under the curve:* Higher AUC of FVII:C was significantly predicted by higher age ( $\beta=.40$ ,  $p=.006$ ;  $\Delta R^2=.11$ ) independent of MAP ( $p=.63$ ) and BMI ( $p=.22$ ). The whole

model including age, BMI, and MAP explained 22% of the variance in FVII:C AUC ( $F(3/59)=5.41$ ,  $p=.002$ ).

*Fibrinogen area under the curve:* Independent of MAP ( $p=.94$ ) and BMI ( $\beta=.44$ ,  $p=.001$ ;  $\Delta R^2=.14$ ) higher age predicted higher fibrinogen AUC ( $\beta=.26$ ,  $p=.041$ ;  $\Delta R^2=.05$ ) the total model explaining 36 % of fibrinogen AUC variance ( $F(3/59)=10.82$ ,  $p<.001$ ).

### 3.3 General linear models

The TSST caused significant increases in fibrinogen ( $p=0.02$ ), but increases in FVII:C ( $p=0.08$ ) and D-dimer ( $p=.14$ ) were not significant across all subjects (Figure 1). To validate the results from the above regression analyses, we applied general linear models with repeated measures of coagulation factors as dependent variables and age as a continuous independent variable. We controlled for BMI and MAP in all analyses. In terms of repeated D-dimer measurements, the main effect of age ( $F(1/59)=23.5$ ,  $p<0.001$ ,  $f=0.61$ ) and the interaction of stress-by-age ( $F(1.5/90.4)=4.36$ ,  $p=0.024$ ,  $f=.33$ ) were significant. In terms of repeated measurements of fibrinogen and FVII:C, the main effects were significant for age (fibrinogen:  $F(1/59)=4.4$ ,  $p=0.04$ ,  $f=0.26$ ; FVII:C:  $F(1/59)=8.4$ ,  $p=0.005$ ,  $f=0.36$ ), whereas the interactions between stress and age were not.

In order to further analyse the interaction between age and stress for D-dimer reactivity, we performed two post-hoc tests. First, we calculated the partial correlation coefficients between age as a continuous variable and stress-induced changes in D-dimer levels (i.e. D-dimer level immediately after stress minus D-dimer level at baseline; D-dimer level 20 min after stress minus D-dimer level at baseline) controlling for BMI and MAP. We found a direct relationship between age and stress change in D-dimer from baseline to immediately after stress ( $r=.26$ ,  $p=.04$ ) and

from baseline to 20 min after stress ( $r=.33$ ,  $p=.01$ ). Second, we addressed the question whether recovery in D-dimer levels was also affected by age. We computed a recovery index, i.e. the difference between D-dimer levels 20 min after stress and D-dimer levels immediately after stress. Again, we calculated the partial correlation coefficient between age as a continuous variable and the recovery index of D-dimer controlling for BMI and MAP, but this association failed to reach statistical significance ( $r=-.18$ ,  $p=.17$ ).

For illustrative purposes, Figure 1 (Panel A-C) shows coagulation responses to the TSST in three groups of subjects with younger age (20-29 yrs,  $n=28$ ), middle age (30-45 yrs,  $n=17$ ) and older age (46-65 yrs,  $n=18$ ).

### **3.4 Association between cortisol and coagulation parameters in relation to age**

*At rest.* Regression analysis controlling for BMI and MAP showed that resting cortisol level did neither significantly predict any of the coagulation parameters ( $p's < .56$ ) nor change the significance of the associations between age and resting levels of coagulation parameters.

*Stress reactivity.* To address whether the associations between age and stress reactivity of the coagulation parameters were related to cortisol secretion, we recalculated all previous regression analyses and general linear models. We entered cortisol AUC as a further predictor into regression analyses and as a further covariate into general linear models. Cortisol AUC was not a significant predictor of any coagulation parameter AUC ( $p's < .39$ ). Likewise, cortisol AUC was not revealed as a significant covariate of any significant main or interaction effect observed in the repeated measures ANCOVA of the three coagulation parameters ( $p's < .39$ ). Thus, controlling for cortisol did not change the significance of the reported regression and GLM results.

#### 4. Discussion

Hemostasis maintains equilibrium through a system of interactions between coagulation factors to protect against hemorrhage. However, anomalies of the coagulation system can have deleterious effects. With aging the incidence of thrombotic cardiovascular disease increases (1, 2). Furthermore, psychological stress elicits net hypercoagulability (20, 22) which might contribute to atherogenesis and thrombogenesis, and ultimately ACS (20). The main objective of the present study was to investigate whether age is associated with coagulation factor levels throughout an acute stress process, i.e. at baseline, immediately following stress, and recovery. Results confirmed that increased age was associated with greater fibrinogen, D-dimer and FVII:C levels between rest until 20 min after acute stress. This suggests that during acute psychological stress, age affects hemostasis. A secondary goal was to examine whether age interacts with stress or, in contrast, whether age and stress are independent variables for increased coagulation factor levels. As expected, age predicted all three coagulation variable levels at rest, and participation in the stress task also increased the three coagulation factors. Previous studies corroborate that age (3, 10, 11, 13) and psychological stress (20, 22) affect coagulation. We found that only D-dimer levels were modulated by age and stress interactions, while age was independent from acute stress for fibrinogen and FVII:C levels. More specifically, there was a direct relationship between age and stress related changes of D-dimer immediately after stress as well as 20 min after stress. This indicates that with increasing age, the stress-induced changes in D-dimer levels linearly increased from baseline to immediately after stress and even until 20 min later. Of potential clinical importance, it has been argued that if stress-induced changes in biological systems are relatively prolonged, this might cause wear and tear –

in this case on the cardiovascular system (36). We further found that the recovery in D-dimer levels from stress was not associated with age. Noteworthy, cortisol secretion did not influence any of the reported results. The latter finding concurs with our previous study in middle-aged men (34)

Our findings suggest that with age fibrin turnover is exaggerated in response to stress, which may increase risk for disorders associated with hemostasis. Prospective studies have demonstrated that D-dimer is associated with the risk of myocardial infarction (14), cerebrovascular events (37) and peripheral arterial disease (38). Whereas for fibrinogen and FVII:C levels, acute stress or aging were additive for increases in these coagulation factors. These results may be important in understanding mechanisms that cause older groups to be vulnerable to stress for developing diseases associated with hemostasis.

Of the hemostatic factors examined, fibrinogen has the strongest evidence for CVD risk prediction (39), and is associated with both atherogenesis and thrombogenesis. One study found that for each standard deviation increase in fibrinogen above the mean, there was an 84% increase in the 5-year risk of ischemic heart disease (40). Although for FVII:C there is less conclusive information on its role in atherothrombogenesis (12), it plays a pivotal role in initiating the extrinsic coagulation pathway, and may play a pathophysiologic role in atherothrombosis. Therefore, the findings of our study that aging and stress independently increase fibrinogen and FVII:C levels may suggest that when older adults experience acute stress there may be additive factors that increase risk of CVD.

Aging is associated with increases in atherogenesis and thrombogenesis, which may be due to alterations in various physiological factors including the vasculature, hemostasis and endothelium, including platelets, coagulation and fibrinolytic factors (3). During aging both



genetic and environmental factors influence hemostasis. A number of polymorphisms in genes related to hemostatic factors have been identified potentially playing a role in late life (41). Also, diet (42, 43) and reduced physical activity of many elderly people may contribute to thrombotic risk (44). Indeed, high D-dimer levels are associated with functional impairment from declines in health associated with aging (45). Although, participants in our study were deemed to be in good physical health, there may be physiological differences with age. For example, tonic whole-body sympathetic nervous system activity increases with age (46), which can subsequently affect physiological parameters in response to stress (47). For instance, sympathetic hormone increases during acute stress are associated with D-dimer levels (48).

Moreover, repeated acute mental stress or chronic stress can induce abnormal central and cardiac autonomic activities to increase catecholamines, heart rate, blood pressure (BP), and platelet activity, resulting in atherosclerosis, and poor left ventricular function, leading to CVD (49). For example, chronic stress confers D-dimer elevation and hypercoagulability risk (29) and interactions between age and chronic stress significantly predicted D-dimer level (29). These findings were similar to the present study with age and acute stress interacting for D-dimer levels, suggesting that repeated acute stress might also increase vulnerability in the elderly for hypercoagulability and subsequent diseases associated with hemostasis.

The present study further suggests that both age and stress are involved in alterations in hemostasis by altering the two systems, coagulation and fibrinolysis since factors from both systems increased with age and acute stress. Our study showed several strengths, which included recruitment of apparently healthy and unmedicated subjects over a broad age range. However, the study does also have its limitations. Our findings were obtained in a sample of apparently healthy men with BP in the normotensive and mildly hypertensive range and may not be

generalized to individuals with more severe hypertension and women. We restricted our study to men for two reasons. First, adult men under the age of 65 have a relative disadvantage compared to women with regard to an increased risk of coronary disease mortality (50). The extent to which this is due to gender differences in hemostasis is still unclear. In a meta-analysis the magnitude of the association between fibrinogen levels and increased coronary risk differs by gender (51). However, studies examining the predictive value of D-dimer and FVII are scarce in women. whereas, in men it was shown that elevated circulating levels of D-dimer (7) and FVII (9) predicted increased risk of coronary disease. A second, reason we restricted this study to men was that resting levels of hemostasis measures (52) and hemostatic responses to stress (23) are shown to be affected by the menstrual cycle. In sum, we found evidence that aging affects coagulation factor alterations in response to acute stress. By virtue of eliciting a proatherogenic profile, we observed that stress could stimulate the atherogenic process even in apparently healthy men. This may further suggest that repeated acute stress might contribute to artery occlusion or thrombosis in coronary patients. Our observations require replication in larger samples and other populations, and the underlying mechanisms remain to be elucidated.

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**Table 1. Characteristics of the 63 subjects studied**

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Age [years]	36.7 ± 1.72 (20 – 65)
Body mass index [kg/m <sup>2</sup> ]	24.8 ± .38 (20.7 – 34.3)
Systolic blood pressure [mmHg]	124.0 ± 1.70 (89.5 – 158.0)
Diastolic blood pressure [mmHg]	80.1 ± 1.27 (60.5 – 97.0)
Mean blood pressure [mmHg]	94.74± 1.33 (71.2 – 116.0)

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Values are given as means ± SEM (range)

### **Legend to Figure 1 (Panels A-C)**

Values are means $\pm$ SEM. Stress reactivity of, D-dimer (Panel A), FVII:C (Panel B), and fibrinogen (Panel C) across all 63 subjects. We applied general linear models with repeated measures of coagulation factors as dependent variables and age as continuous independent variable while controlling for BMI and MAP were in all analyses. In terms of D-dimer measurements, the main effect of age ( $F(1/59)=23.5$ ,  $p<0.001$ ,  $f=0.61$ ) and the interaction of stress-by-age ( $F(1.5/90.4)=4.36$ ,  $p=0.024$ ,  $f=.33$ ) were significant (Panel A). In terms of fibrinogen (Panel C) and FVII:C (Panel B), the main effects were significant for age (fibrinogen:  $F(1/59)=4.4$ ,  $p=0.04$ ,  $f=0.26$ ; FVII:C:  $F(1/59)=8.4$ ,  $p=0.005$ ,  $f=0.36$ ), whereas the interactions between stress and age were not. The figure shows these data for illustrative purposes by three categories of age.

**Figure 1. Hemodynamic levels in three groups of subjects with younger (20-29yrs, n=28), middle (30-45 yrs, n=17) and older (46-65 yrs, n=18) age over time**

