Increased fibrinogen responses to psychophysiological stress predict future endothelial dysfunction implications for cardiovascular disease?

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Abstract:

Stress influences the risk of cardiovascular disease. Acute mental stress can induce both low-grade inflammation and endothelial dysfunction. The relationship between inflammatory responses to stress and future endothelial function is unexplored. Knowledge on the impact of other cardiovascular risk factors, such as dyslipidaemia, on such relationships is also limited We investigated the relationship between inflammatory responses to an acute mental stress challenge and endothelial function plus the influence of dyslipidaemia on the associations. Interleukin-6 (IL-6), tumor necrosis factor α (TNFα) and fibrinogen were assessed at baseline, immediately following standardized behavioural tasks and 45 minutes post-task in 158 participants. Blood pressure and heart rate responses were measured. Flow-mediated dilatation (FMD) was measured 3 years later. Fibrinogen and IL-6 increased post-stress (p=<0.001 &0.003) but TNFα was unchanged (p=0.09). An independent negative association between FMD and change in fibrinogen at 45 minutes (β=-0.047 p=0.016) remained after multiple adjustment (baseline fibrinogen, baseline diameter, reactive hyperaemia, age, gender and other cardiovascular risk factors). There was no association between FMD and change in IL-6 or TNFα. There were no differences in the responses to stress between those with and without dyslipidaemia. However, there was an interaction between the presence of dyslipidaemia and immediate change in fibrinogen with stress which was associated with FMD. Those participants with dyslipidaemia who had a greater change in fibrinogen had lower FMD. We conclude that elevated fibrinogen responses to stress are associated with future endothelial dysfunction which may reflect increased cardiovascular risk.

1. Introduction:

There is substantial evidence for an association between psychosocial stress and the development of cardiovascular disease, which has led to it being considered as an important cardiovascular risk factor (Everson-Rose and Lewis, 2005; Steptoe and Kivimaki, 2013; Yusuf et al., 2004). However, despite recent advances, the pathophysiological pathways connecting the two are not yet fully understood. Assessment of the dynamic cardiovascular and inflammatory responses to acute mental stress challenges provides the opportunity to understand more fully the potential mechanisms by which everyday psychological stresses influence the development of cardiovascular pathophysiology. In turn this may improve our understanding of how stress influences the presentation of clinical disease and may offer potential novel targets for treatment.

Inflammation plays a key role in the initiation, development and destabilisation of atherosclerotic plaques (Hansson et al., 2015). Low grade systemic inflammation is associated with adverse cardiovascular risk in those with and without cardiovascular disease (Liuzzo et al., 1994; Ridker et al., 1997). Acute stressors trigger inflammatory responses which may play a role in the pathogenesis of cardiovascular disease (Steptoe and Brydon, 2009; Steptoe et al., 2007).

Endothelial vasomotor function is a well-established measure of general vascular health. Flow-mediated dilatation (FMD), a non-invasive measure of endothelial function, is diminished in the presence of traditional cardiovascular risk factors and also in the setting of inflammatory conditions (Celermajer et al., 1994; Celermajer et al., 1992; Di Minno et al., 2015; Woo et al., 2004). Furthermore, both acute inflammation, for example post vaccination, and acute mental stress have been shown to cause transient impairment of endothelium dependent dilatation in otherwise healthy people (Ghiadoni et al., 2000; Hingorani et al.,

2000; Spieker et al., 2002). This may be due to the effects of inflammatory cytokines and fibrinogen which activate the endothelium, reducing nitric oxide bioavailability (Hansson et al., 2015; Tousoulis et al., 2011). We have also previously shown that those with the most pronounced inflammatory responses (interleukin-6 [IL-6], tumor necrosis factor α [TNFα] and fibrinogen) to acute mental stress had greater arterial stiffness and increases in ambulatory systolic blood pressure when assessed three years later (Brydon and Steptoe, 2005; Ellins et al., 2008). Therefore the inflammatory response to acute mental stress characterised in this way could serve as an individual biomarker of risk for the development of endothelial dysfunction and subsequent cardiovascular events.

Dyslipidaemia is associated with increased cardiovascular risk and endothelial dysfunction as well as a raised inflammatory profile (Andersson et al., 2014; Celermajer et al., 1992; Ueland et al., 2006). There is limited work looking at how responses to acute stress might be further influenced by the presence of cardiovascular risk factors such as dyslipidaemia. One small study in men with mixed dyslipidaemia saw no difference in the haemodynamic responses to acute stress challenges compared to controls with normal lipids (McCann et al., 1995). However, subjects with type II diabetes have been shown to have blunted blood pressure, heart rate and IL-6 responses to an acute mental stress (Steptoe et al., 2014). Studies looking at the effect of the presence of risk factors on the endothelial response to stress are also few in number and small in size, with mixed findings. They also do not look at how responses to stress might influence endothelial function over the longer term (Cardillo et al., 1998; Ghiadoni et al., 2000) . We have previously shown that greater Fibrinogen and TNFα responses following a modest acute pyschophysiological stress stimulus was associated with increased carotid arterial stiffness, a measure of structural arteriosclerotic/atherosclerotic changes, in middle aged civil servants. The relationships between inflammatory responses to acute mental stress and future endothelial function, a more dynamic measure of arterial

(patho)physiology than arterial stiffness and the potential influence of dyslipidaemia have not been investigated. The aim of this study was (a) to investigate associations between inflammatory responses to acute mental stress and endothelial function assessed at three years, and (b) to evaluate the potential influence of an adverse lipid profile on this association.

2. Materials and Methods:

2.1. Participants

The Whitehall II epidemiological cohort consisted of 10,308 nonindustrial civil servants aged 35 to 55, who were recruited between 1985 and 1988 to investigate social and occupational influences on health and disease. Follow-up of these participants has occurred through clinic visits and self-administered questionnaires every 2-5 years. Between 1999 and 2000, 123 men and 105 women from the Whitehall II Study underwent psychophysiological testing as part of a psychobiology sub-study (Marmot et al., 1991; Steptoe et al., 2002a). Participants within the sub-study were of white European origin, aged 45-59 years, lived in London, and were in full time work, with no history or indicators for coronary heart disease or hypertension. Selection had been stratified by employment grade to ensure a wide range of socio-economic status. 158 participants (52±3 years) underwent endothelial function assessment during Phase 7 of the cohort study, 3 years after psychophysiological stress testing.

2.2 Psychophysiological stress testing

Studies took place in the morning or afternoon in a temperature-controlled laboratory. Participants were asked to refrain from drinking alcohol or exercising on the evening before or the day of testing, and to not drink caffeine or smoke for 2 hours prior to the study. Blood pressure and heart rate were continuously monitored during the study using a Partapress-2

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(Finapress Medical Systems, Amsterdam, NL). Participants rested for 30 minutes following the insertion of a cannula for blood sample collection. During the last 5 minutes of the rest period, baseline blood pressure and heart rate were recorded and a baseline blood sample was drawn. Following this, two moderately stressful tasks were administered in a random order with a 5 minute inter-task interval. These tasks (computerized colour-word interference task and mirror tracing) have previously been used in cardiovascular stress research (Jennings et al., 2004). The rationale for using these tasks is explained elsewhere (Steptoe and Marmot, 2002).

The two tasks each lasted 5 minutes. A second blood sample was taken immediately post the second task and participants were left to rest quietly, reading or watching wildlife videos. Two 5 minute post-stress blood pressure and heart rate recordings were made at 15-20 minutes and 40-45 minutes. A final blood sample was taken after 45 minutes. The study was approved by the UCL/UCLH Committee on the Ethics of Human Research.

2.3 *Blood assays*

Blood samples were collected in EDTA tubes and serum gel tubes, and centrifuged immediately at 2500 rpm for 10 min at room temperature. The plasma was removed and stored at -80 °C until analysis. We have shown in previous studies that fibrinogen responds immediately to psychological stress, while increases in IL-6 and TNFα emerge after 30–45 min (Steptoe et al., 2007; Steptoe et al., 2003). Fibrinogen was therefore assayed from all three samples, whilst IL-6 and TNFα were only assessed from the baseline and 45 min post-stress samples. C-reactive protein (CRP) was measured from baseline samples only. Clottable fibrinogen was measured by an automated Clauss assay in a MDA-180 coagulometer (Oragon Teknika, Cambridge, UK). The coefficient of variation (CV) was <8%. IL-6 and TNFα were measured using high sensitivity two-site ELISAs from R&D Systems (Oxford,

UK). The limit of detection of the human TNFα assay was 0.10 pg/ml and intra- and inter-assay CVs were 6.9% and 8.4%. For IL-6, the limit of detection was 0.09 pg/ml, and intra- and inter-assay CVs were 5.3% and 9.2%. CRP was detected using a sensitive, two-site ELISA with antibodies from Dako diagnostics (Ely, Cambs, UK). The inter- and intra-assay CVs were 2.5% and 4.1%.

The serum was snap frozen at -70°C until analysis. Samples were taken at baseline only. Total cholesterol and triglycerides were measured in a centrifugal analyser by enzymatic colorimetric methods and HDL cholesterol was determined after dextran sulphate-magnesium chloride precipitation of non-HDL cholesterol. LDL was computed using the Friedewald equation.

2.4 Other measures

Height, weight, waist and hip circumference were assessed and used to calculate body mass index (BMI) and waist/hip ratio. Socio-economic status (SES) based on current or last known grade of employment was determined by questionnaire as was smoking status.

2.5 Endothelial function assessment

Flow-mediated dilatation was assessed in the right brachial artery using high resolution ultrasound (Prosound 5500 ALOKA) as previously described (Donald et al., 2006). Analysis of changes in brachial artery diameter was done using automated edge detection software (Brachial Tools, Iowa City, Iowa). FMD was expressed as absolute change in diameter from baseline. Reactive hyperaemia was calculated from the baseline and maximal velocity time integral in the first 15 seconds following cuff release and expressed as a percentage.

2.6 Statistical analysis

SPSS version 20 was used for the analyses. Data were checked for normality and those non-normally distributed variables were transformed using ln transformation. Five participants were excluded from the analysis as they had starting taking lipid or blood pressure medication since undergoing stress testing. Differences between baseline characteristics between males and females were tested using independent samples t-tests or the independent samples Mann Whitney U test. Chi square was used to assess for differences between smoking and SES status. Relationships between FMD and conventional risk factors were assessed using partial correlations adjusted for age, gender, baseline diameter and reactive hyperaemia. Haemodynamic and inflammatory responses to psychological stress testing were assessed using repeated measures analysis of variance with trial as the within-subject factor. Post-hoc comparisons were made using Tukey's least significant differences (LSD) test. Gender was then added to the model as a between subject-factor to investigate whether there were differences in the stress responses between the two groups.

Presence of associations between endothelial function and inflammatory responses were investigated using multiple linear regression. Adjustments were made for baseline inflammatory variable, baseline brachial artery diameter, reactive hyperaemia, age, gender, BMI, waist/hip ratio, systolic and diastolic blood pressure, HDL and LDL cholesterol, glucose, socioeconomic status and smoking.

2.6.1 Dyslipidaemia

Participants were classified as having dyslipidaemia if they met one of the following criteria. Total cholesterol ≥ 6 triglycerides ≥ 1.7 HDL <1 LDL≥4 (mmol/L). Comparisons were made between those with and without dyslipidaemia using independent t-tests. Repeated measures ANOVAs were used including dyslipidaemia as a between subject-factor to investigate

whether dyslipidaemia influenced the responses to stress. To investigate whether the presence of dyslipidaemia and the inflammatory response influenced future endothelial function the effect of interactions between dyslipidaemia and change in each inflammatory variable in response to stress on FMD were tested using linear regression. Baseline diameter, reactive hyperaemia and baseline inflammatory variable were also included in the analysis.

3. Results:

3.1 Participant characteristics

Characteristic	All (n=153)	Male (n=84)	Female (n=69)	p
Age (yrs)	52 ± 3	53 ± 3	52 ± 3	*0.021*
Smoker (Y)	9 (5.9%)	6 (7.1%)	3 (4.2%)	*0.47*
SES				
Higher	60 (39.2%)	34 (40.5%)	26 (37.7%)	*0.84*
Intermediate	50 (32.7%)	28 (33.3%)	22 (31.9%)	
Lower	43 (28.1%)	22 (26.2%)	21 (30.4%)	
BMI (kg/m2)	25.23 ± 3.52	25.36 ± 3.25	25.08 ± 3.85	*0.63*
Waist/hip ratio	0.84 ± 0.09	0.90 ± 0.07	0.78 ±0.06	*<0.001*
SBP(mmHg)	114 ± 12	118 ± 11	109 ± 12	*<0.001*
DBP(mmHg)	69 ± 9	71 ± 9	67 ± 9	*0.005*
HR(bpm)	64 ± 8	64 ± 9	66 ± 8	*0.14*
TC(mmol/L)	5.38 ± 0.87	5.42 ± 0.84	5.32 ± 0.91	*0.47*
Trigs (mmol/L)	1.34 ± 0.71	1.45 ± 0.65	1.21 ± 0.76	*0.003*
HDL (mmol/L)	1.57 ± 0.39	1.43 ± 0.30	1.74 ± 0.41	*<0.001*
LDL (mmol/L)	3.20 ± 0.81	3.34 ± 0.79	3.02 ± 0.82	*0.018*
Total/HDL	3.65 ± 1.13	3.96 ± 1.01	3.24 ± 1.13	*<0.001*
Glucose (mmol/L)	5.30 ± 0.79	5.34 ± 0.72	5.25 ± 0.88	*0.54*
CRP (mg/L)	1.02 ± 1.29	0.92 ± 0.97	1.13 ± 1.59	*0.8*
BL dia (mm)*	3.60 ± 0.71	4.02 ± 0.61	3.08 ± 0.41	*<0.001*
FMD (mm)*	0.19 ± 0.10	0.19 ± 0.10	0.20 ± 0.10	*0.81*
RH% (%)*	658 ± 260	648 ± 249	665 ± 270	*0.61*

Table 1: Participant characteristics for all and by gender. SES= socio-economic status; BMI= body mass index; SBP = systolic blood pressure; DBP= diastolic blood pressure; HR=heart rate; TC=total cholesterol; Trigs=triglycerides; HDL=high density lipoprotein; LDL=low density lipoprotein; CRP= C-reactive protein; BL dia= brachial artery baseline diameter; FMD=flow-mediated dilatation; RH%= Reactive hyperaemia as a percentage. *Assessed at phase 7.

Table 1 shows the characteristics for those participants included in the study. Males were older with a greater waist/hip ratio, blood pressure, triglycerides, LDL, Total/HDL ratio and brachial artery diameter, whilst HDL was significantly lower (p<0.03).

3.2 Associations with conventional risk factors

FMD was correlated with diastolic blood pressure (DBP) and heart rate (HR) following adjustment for age, gender, baseline diameter and reactive hyperaemia (DBP r= 0.18 p=0.037 & HR r=0.23 p =0.007). No other conventional risk factors, including systolic blood pressure, BMI, waist/hip ratio, total cholesterol, LDL, HDL, triglycerides, glucose and CRP, were associated with FMD. In addition there were no significant correlations between FMD and baseline IL-6, TNFα or fibrinogen.

3.3 Responses to acute mental stress

Systolic- and diastolic-BP and HR all increased in response to the mental stress challenge (all p<0.001). Both fibrinogen and IL-6 increased post-stress (p <0.001 & 0.003 respectively). The increase in TNFα levels post stress did not reach significance (p= 0.09) (Table 2). There were no differences in the responses by gender except for TNFα (F(1,140) 4.0 p=0.047). This was due to an increase in TNFα in men (baseline 2.19 pg/ml vs post-stress 2.31 pg/ml) and a decrease in women (baseline 2.04 pg/ml vs post-stress 2.02 pg/ml).

	Baseline	Stress tasks	Recovery 1	Recovery 2	F	p
SBP (mmHg)	114	138*	119*≠	119*≠	250.3	*<0.001*
DBP (mmHg)	69	83*	73*≠	74*≠	268.3	*<0.001*
HR (bpm)	65	72*	62*≠	63*≠°	234.7	*<0.001*
Fbg (g/L)	2.81	2.87*		2.85*	13.4	*<0.001*
IL-6 (pg/ml)	1.12			1.20*	9.2	*0.003*
TNFα (pg/ml)	2.12			2.18	2.9	*0.09*

Table 2: Haemodynamic and inflammatory responses to a psychophysiological challenge. Geometric means shown for IL-6 and TNFα and arithmetic means for all other measures. Values that have different superscripts are significantly different *= from baseline, ≠=from task, O=from recovery1 (p<0.05) SBP = systolic blood

pressure; DBP= diastolic blood pressure; HR=heart rate; Fbg=fibrinogen; IL-6=interleukin-6; TNFα= tumor necrosis factor α.

3.4 Change in inflammatory markers and association with future endothelial function
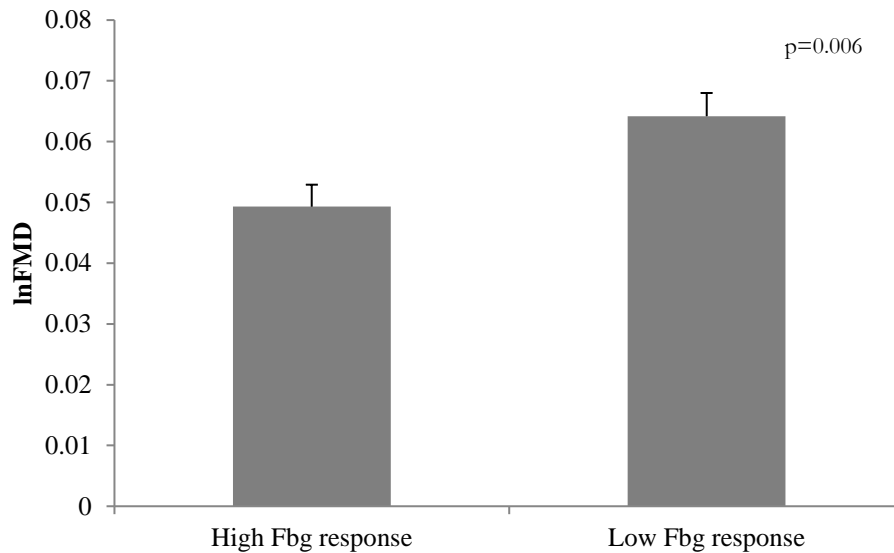

Figure 1: FMD (natural log) by high and low fibrinogen (fbg) response 45 minutes after an acute mental stress challenge, adjusted for baseline arterial diameter, baseline fibrinogen and reactive hyperaemia. Data are mean ± standard error.

To investigate the influence of the haemodynamic and inflammatory responses to an acute mental stress challenge on future endothelial function multiple linear regression modelling between FMD and haemodynamic and inflammatory variables was used. Changes in heart rate and blood pressure post-stress were not associated with endothelial function (data not shown). However, there was a significant negative association between FMD and elevated fibrinogen at 45 minutes after adjustment for baseline arterial diameter and reactive hyperaemia (β= -0.038 [0.018] p= 0.041). The relationship remained after adjustment for baseline fibrinogen, age and gender (β= -0.037 [0.018] p= 0.048), and after the addition of cardiovascular risk factors , waist/hip ratio, BMI , SBP, DBP, glucose, HDL and LDL (β= -0.046 [0.019] p= 0.017). This association was still present after the addition of socio-

economic status and smoking status to the model (β= -0.047 [0.019] p= 0.016), indicating that those participants with a greater change in fibrinogen at 45 minutes had lower FMD 3 years later. There were no relationships between FMD and the other inflammatory measures.

3.5 Dyslipidaemia, inflammatory responses to acute mental stress and endothelial function

62 (21 females) participants met the criteria for dyslipidaemia, with the majority of participants having only one abnormal lipid variable (see supplemental table 1). Those with dyslipidaemia were more likely to be male (p=0.025) and to have greater BMI, waist hip ratio and CRP levels (p<0.001). There was no difference in FMD between those with and without dyslipidaemia (p=0.75).

There were no differences in haemodynamic and inflammatory responses to the psychophysiological stress challenge between the two groups (p>0.05).

There were no significant interactions between dyslipidaemia and inflammatory responses to stress for FMD for the majority of the variables (data not shown) except change in fibrinogen immediately after stress (β=-0.080 [0.019] p=0.024). Further exploration identified that in those with dyslipidaemia a greater change in fibrinogen was associated with lower FMD (β=-0.053 [0.022] p=0.018). There was no association between the immediate fibrinogen response to stress and FMD in those with normal lipids (β=0.000 [0.015] p=0.99).

4. Discussion:

The main finding of this study is that those participants who had a higher fibrinogen response to a psychophysiological stress challenge had less well preserved endothelial function when assessed 3 years later following adjustment for key relevant cardiovascular risk factors. This complements our previous analyses in this cohort which found increased arterial stiffness and

ambulatory blood pressure in those participants who had increased inflammatory responses to a stress challenge (Brydon and Steptoe, 2005; Ellins et al., 2008).

Endothelial dysfunction is an early indicator of atherogenesis. This can be assessed by FMD which provides a measure of change in vasomotor tone caused by the local release of nitric oxide (NO) stimulated by the increase in shear stress during a standardised post-ischaemic hyperaemic response. A poor FMD has been associated with numerous cardiovascular risk factors and an increased risk of cardiovascular events (Celermajer et al., 1994; Celermajer et al., 1992; Clarkson et al., 1996; Green et al., 2011; Woo et al., 2004). Acute mental stress causes endothelial dysfunction both during a stress challenge and immediately afterwards but the mechanisms for this are not yet fully understood (Eriksson et al., 2007; Ghiadoni et al., 2000). Inhibition of cortisol and the endothelin-A receptor have both prevented the impairment of FMD induced by mental stress suggesting potential roles for both of these factors (Broadley et al., 2005; Spieker et al., 2002).

 Inflammation plays a major role in the initiation, development and progression of atherosclerosis and is associated with endothelial dysfunction (Hansson et al., 2015). Although acute inflammation and acute mental stress have been shown to cause acute endothelial dysfunction there is limited work investigating whether and to what extent the acute inflammatory response to mental stress is implicated in the associated endothelial dysfunction (Clapp et al., 2004; Hingorani et al., 2000). One study by Ghiadoni et al measured the inflammatory markers IL-6, IL-1 & TNFα at baseline and 60 mins post stress challenge but did not see a significant change in these markers or any relationship between cytokine levels and FMD or change in FMD (Ghiadoni et al., 2000). Therefore our study linking fibrinogen at 45 minutes to FMD after three years is the first study to show an association between an inflammatory response to acute mental stress and future endothelial function. There were no relationships between FMD assessed 3 years later and any of the

inflammatory measures assessed prior to the mental stress challenge. This may be due to the relatively healthy nature of the cohort with a low prevalence of obesity and other cardiovascular risk factors which are commonly associated with low grade inflammation and endothelial dysfunction.

Fibrinogen, an acute phase protein induced by IL-6 in the inflammatory pathway and a major component of the coagulation cascade, is associated with increased risk of coronary heart disease and stroke (Danesh et al., 2005). It has been implicated in the development of vascular dysfunction and atherosclerosis through its effects on plaque composition, blood viscosity, endothelial and smooth muscle cell activation, platelet aggregation and activation, and immune cell recruitment (Lominadze et al., 2010; Tousoulis et al., 2011).

Increased fibrinogen may affect endothelial function both by mechanical and biochemical processes. Elevated fibrinogen increases blood viscosity which raises shear stress and activates endothelial cells (Davies et al., 2003; Lominadze et al., 2010; Lowe et al., 1997). This stimulates expression and activation of adhesion molecules and integrins, resulting in the attraction and adhesion of monocytes to endothelial cells and the greater production of vasoconstricting agents which may further affect endothelial function and vascular tone (Languino et al., 1993; Lominadze et al., 2005; Suehiro et al., 1997).

Vascular injury triggers the coagulation cascade which results in fibrinogen being converted to fibrin which in turn forms a thin monolayer covering the damaged area. This layer attracts platelets which are also activated by fibrinogen causing further platelet aggregation, inflammatory responses and endothelial dysfunction. As the injury heals the platelets can also become part of the developing lesion/plaque (Badimon, 2014). Therefore elevated levels of fibrinogen through reactions to acute stressors could further exacerbate this process. The lack of association between IL-6 and future endothelial function may suggest that it is these

haemostatic/prothrombotic properties of fibrinogen that could be more important in this setting than its inflammatory properties.

It was interesting that an independent association between FMD was only seen with change in fibrinogen at 45 minutes post stress testing and not with change in fibrinogen immediately after the stress tests. This may indicate that the duration of exposure to a raised fibrinogen may be more important, reflecting more sustained activation of its haemostatic/prothrombotic and proinflammatory affects adversely influencing endothelial function over the longer term. Further studies looking at the time course of the fibrinogen response with more frequent assessment of fibrinogen levels over a longer period would allow a more detailed investigation of this relationship, although such a study could pose practical and ethical challenges in humans.

Gender did not appear to influence the responses to acute mental stress except for TNFα which only increased in men and reflects previous findings (Steptoe et al., 2002b), but TNFα stress responses were not associated with endothelial function.

The presence of dyslipidaemia did not appear to have any influence on the haemodynamic or inflammatory responses to an acute mental stress challenge. Although a little surprising, the severity of dyslipidaemia was only mild. However, when examining interactions between the presence of dyslipidaemia and inflammatory responses to stress and their effect on endothelial function, a significant interaction between dyslipidaemia and change in immediate fibrinogen at stress and FMD was found. Further exploration of this association showed that those participants with dyslipidaemia and a high fibrinogen response to stress had lower FMD. Dyslipidaemia has previously been shown to be associated with poorer endothelial function but the additional presence of high levels of fibrinogen with its haemostatic and prothrombtic effects, even for a short period of time, may exacerbate the

development of endothelial function in this group with mild dyslipidaemia. Considered together with our observation of an independent relationship between fibrinogen at 45 minutes and FMD the evidence of an interaction between dyslipidaemia and immediate fibrinogen response highlights the potential importance of this effect of stress on vascular pathophysiology. Further studies exploring the influence of fibrinogen stress responses and vascular outcomes in selected populations with dyslipidaemia and other cardiovascular risk factors would be valuable.

There are a number of limitations to this study. Endothelial function was not assessed at the time of stress testing so it is unknown whether those participants with poorer FMD 3 years later already had endothelial dysfunction at the point of stress testing. The population were originally selected for being free of cardiovascular disease so the dyslipidaemia group mainly consists of those with mild lipid abnormalities. More adverse lipid profiles such as in familial hypercholesterolaemia may well give different findings. Additionally, blood samples for the mental stress study were not taken in a fasted state as the study protocol did not state the need for a pre-specified prior fast. Therefore, some participants may have been classified as dyslipidaemic due to elevated triglycerides which may have been within the normal range if the participant had fasted. However, the important association between non-fasting triglycerides (and also LDL) and cardiovascular outcomes is well recognised and suggests it is not inappropriate to select participants on the basis of non-fasting triglycerides (Nordestgaard and Varbo, 2014). In addition it may also more accurately reflect an individual's usual waking status as they would rarely be in a fully fasted state. It is also worth noting that although 36 participants had elevated triglycerides within this population, 21 of these individuals would also have been classified as having dyslipidaemia due to their TC, LDL or HDL levels.

Studies were carried out in both morning and afternoon sessions. Although we cannot exclude an influence of circadian rhythm, including an adjustment in our analyses for the time of day did not alter the relationship between fibrinogen responses and FMD. Furthermore, inflammatory measures were not associated with the time of day in a previous analysis of this dataset (Steptoe et al., 2002b).

Measures of plasma adrenaline and noradrenaline were not assessed as venous measures of these hormones can be unreliable indicators of general activation of the sympathetic nervous system. In addition the half life of catecholamines in plasma is 1-2 min, and venous concentrations in the forearm have been shown to be primarily due to local muscle activity (Hjemdahl, 1993). In order to assess dynamic autonomic function more reliably would have required complex, invasive and costly techniques which were not included in this protocol. However, significant increases in both blood pressure and heart rate in response to the tasks were seen in this study, which alongside self-reported measures of perceived stress previously reported in this cohort (Steptoe and Marmot, 2002) indicated the stressful nature of these challenges.

These data are from a post-hoc analysis of the Whitehall II psychobiology study and the results are consistent with previously published analyses from this study showing a relationship between fibrinogen stress responses and large artery stiffness, strengthening the implications of those findings. We are unaware of similar cohorts in which an attempt to replicate these findings could be made at present, but we believe our results should encourage further prospective research to investigate further these potentially important relationships between stress, traditional risk factors and cardiovascular disease.

In conclusion participants with an elevated fibrinogen response at 45 minutes post-stress and those with dyslipidaemia who had a greater immediate fibrinogen response to stress had

impaired endothelial function 3 years later. These results implicate the fibrinogen response to stress as a pathway through which psychological factors may contribute to the development of cardiovascular disease.

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Supplement

	Dyslipidaemia categories				**Nº of categories met**		
	TC≥6	**Trigs ≥1.7**	**HDL <1**	**LDL ≥4**	**1**	**2**	**3**
Yes	40	36	2	33	27	21	14
No	112	116	150	119			

Supplement table 1: Number of participants who met each category for dyslipidaemia and the

number of participants who met one or more of the categories. TC=total cholesterol;

Trigs=triglyceride; HDL=high density lipoprotein; LDL=low density lipoprotein.