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**Elevated levels of circulating matrix metalloproteinase-9 are associated with a dysregulated cortisol rhythm - a case-control study of coronary artery disease.**

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## **Abstract**

A dysregulated cortisol pattern has been found to be associated with systemic inflammatory activity in patients with coronary artery disease (CAD). Matrix metalloproteinase (MMP)-9 is involved in both inflammation and matrix degradation and considered a main contributor to coronary plaque rupture. In this study, we hypothesized that a dysfunctional cortisol response also involved a failure to regulate systemic MMP-9 levels in CAD patients. Total MMP-9, active MMP-9 and the endogenous inhibitor TIMP-1 were measured in 30 CAD patients and 30 healthy controls. Morning and evening cortisol was measured in repeated saliva samples. Patients had higher levels of total and active MMP-9 (both  $p < 0.01$ ) and increased 24-hour cortisol output ( $p < 0.05$ ) characterized by higher levels of evening cortisol ( $p = 0.011$ ). MMP-9 was associated with evening cortisol ( $p < 0.001$ ) independent of smoking and inflammatory markers. Compared with controls, patients also showed a blunted cortisol response to stress. After stress, the levels of MMP-9 became significantly reduced in controls whereas they remained unchanged in patients. The data indicate that MMP-9 is differently regulated in patients due to a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis and emphasize the role of MMP-9 as a possible link between stress and cardiovascular disease.

**Key words:** Matrix metalloproteinase; Coronary artery disease; Cortisol; Stress.

## **Introduction**

Matrix metalloproteinase (MMP)-9, a major physiological mediator of matrix degradation and inflammatory activity, is assumed to play an important role in the progress of atherosclerosis (Galis and Khatri, 2002). The expression of MMP-9 is enhanced in rupture-prone atherosclerotic plaques and circulating levels of MMP-9 have been reported to be elevated in patients with coronary artery disease (CAD), mainly in those with unstable conditions of the disease (Kai et al., 1998; Tayebjee et al., 2005). MMP-9 has also been identified as a novel predictor of future cardiovascular events in patients with existing CAD, thus suggesting its potential role as both diagnostic and prognostic biomarker in CAD (Blankenberg et al., 2003; Jefferis et al., 2009).

Proinflammatory cytokines are implicated in the transcriptional control of MMP-9 and its endogenous tissue inhibitor (TIMP-1) (Zhang et al., 1998; Harkness et al., 2000). Glucocorticoids may suppress MMP-9 by causing a shift of cytokine production from a pro-inflammatory to an anti-inflammatory pattern but they are also capable of down-regulating MMP-9 in a direct way (Harkness et al., 2000; Aljada et al., 2001). On the basis of both clinical and experimental studies, it has become evident that a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis involves a failure to counteract inflammatory activity (Webster et al., 2002). Our own group has shown that a dysregulated pattern of cortisol secretion, characterized by a flattened diurnal rhythm of cortisol and an attenuated cortisol response to stress, is associated with systemic inflammatory activity in patients with CAD (Nijm et al., 2007).

One intriguing possibility is that a dysfunctional cortisol response also involves a failure to regulate MMP-9 expression. In the present study, we investigated a) whether circulating levels of total MMP-9, active MMP-9 and TIMP-1 differed between clinically stable CAD patients and their matched healthy controls and b) if these levels were associated with dynamic changes in salivary cortisol.

## **Methods**

Thirty patients (< 70 years) were assessed 12 –14 weeks after a first-time acute myocardial infarction. Thirty individuals, randomly selected from the population register, served as controls. The protocol has been described in detail elsewhere (Nijm et al., 2007). The participants were instructed to collect saliva during two 3-day periods. The first sample was taken 30 minutes after awakening and the second sample in the evening before going to bed. Saliva was collected with Salivette cotton swabs (Sarstedt, Nümbrecht, Germany) and immediately frozen at -20 C° until analysis. The participants also underwent a psychological stress test, including two kinds of stressors (anger recall followed by

arithmetic test). The stress test always started at 07.30 AM after a 12-hour fast. Repeated saliva samples were collected before and up to 34 minutes after stress. Serum samples were collected before the test (day 1) and the next morning (day 2). The study was originally designed to detect stress-induced differences in CRP. In humans, CRP has been shown to reach a peak level at 21 hours after the cessation of IL-6 infusion (Steensberg, 2003) and therefore, we chose to collect the post-stress serum sample after 24 hours.

Free cortisol was determined in a 24-hour urine sample and in saliva by a modified commercial radioimmunoassay (Diagnostic Products Corporation, Los Angeles, US). Serum samples were assayed for total MMP-9 and TIMP-1 using ELISA immunoassays (Quantikine HS, R & D Systems Europe Ltd, Abingdon, Oxon United Kingdom). The lower limits of detection for MMP-9 and TIMP-1 were 0.31 and 0.16 ng/ml, respectively. The interassay coefficient of variation was < 5 % for MMP-9 and TIMP-1. Active MMP-9 was measured by a fluorometric assay (Fluorokine E, R & D Systems Europe Ltd) with a lower limit of detection of 0.25 ng/ml and an interassay coefficient of variation of < 10 %. Serum samples were also assayed for CRP (Roche Diagnostics GmbH, Vienna, Austria) and IL-6 (Quantikine HS, R & D Systems Europe Ltd).

Data were analysed using SPSSPC (SPSS, Inc., Chicago, Illinois). Wilcoxon signed-rank test was used to analyse differences within the groups and two-way repeated ANOVA for comparisons between groups. Spearman rank correlation test was used to evaluate the relationships between variables and a multiple linear regression analysis to investigate interactions between MMP-9, TIMP-1, cortisol, smoking, white blood cell counts, CRP and IL-6. Data are given as median (25th, 75th percentile).

## Results

Clinical characteristics and laboratory variables including white blood cell counts, CRP, IL-6 and 24-hour cortisol secretion are outlined in Table 1. All patients were using various combinations of nitrates, beta-blockers and/or calcium antagonists and 73 % were on long-term therapy with statin. As shown in Table 1, no significant differences between patients and controls were seen except for a higher diastolic blood pressure in controls and a higher total cortisol output in patients. The morning cortisol levels were similar in patients and controls, 11.1 (10.0, 13.0) vs 11.2 (10.1, 14.3) nmol/l while the evening cortisol levels were significantly higher in patients, 4.1 (2.4, 6.8) vs 1.4 (1.1, 4.6),  $p = 0.011$ . Before stress, salivary cortisol did not differ between patients and controls, 8.6 (6.6, 12.6) vs 10.4 (6.6, 12.3) nmol/l. However, despite similar cardiovascular responses, the patients attained significantly lower cortisol levels compared with controls after stress, 9.3 (7.8, 13.5) vs 13.5 (12.0, 17.0) nmol/l ( $p < 0.01$ ). As has been reported previously (Nijm et al, 2007), the rise in cortisol after

stress (34 minutes) was inversely correlated to the rise in CRP after stress (24 hours),  $r = -0.41$ ,  $p < 0.01$ .

As shown in Table 2, the levels of total and active MMP-9 were significantly higher in patients compared with controls on both test occasions while TIMP-1 levels did not differ. The levels of MMP-9 and TIMP-1 were not significantly influenced by clinical characteristics, like smoking, body mass index or blood pressure. On both occasions, total and active MMP-9 were correlated to white blood cell counts (both  $p < 0,01$ ) and IL-6 (both  $p < 0,05$ ) but not to CRP. TIMP-1 was correlated to IL-6 ( $p < 0,05$ ) but not to white blood cell counts or CRP. The levels of total MMP-9, active MMP-9 and TIMP-1 were all significantly associated with evening cortisol ( $r = 0,46$ ,  $p < 0,001$ ,  $r = 0,42$ ,  $p < 0,01$ , and  $r = 0,64$ ,  $p < 0,001$ , respectively). After adjustment for smoking, white blood cell counts, CRP and IL-6, total and active MMP-9 remained associated with evening cortisol (standardized  $\beta = 0,52$  and  $0,43$  respectively,  $p < 0.001$ ) while TIMP-1 lost its significant relationship with cortisol.

In controls, the levels of total MMP-9, active MMP-9 and TIMP-1 significantly decreased from day 1 to day 2 while the levels remained unchanged in patients (Table 2). The cortisol response was not significantly associated with any changes in MMP-9 or TIMP-1 after stress except for active MMP-9 that showed an inverse correlation with the cortisol peak at 34 minutes ( $r = -0.53$ ,  $p = 0,013$ ).

## Discussion

The findings of high levels of total and active MMP-9 in CAD patients agree with previous studies (Kai et al., 1998; Tayebjee et al., 2005). In most reports, the interest of MMP-9 in CAD has focused on patients with acute myocardial infarction in whom the elevation of serum MMP-9 have shown a direct relation to the presence of plaque rupture (Fukuda et al., 2006). The increased levels in CAD patients may thus reflect enzyme activity in atherosclerotic tissue but peripheral leukocytes are a potential source of MMP-9 as well. The enzyme is assumed to participate in several stages of atherosclerosis involving leukocyte adhesion, cell migration and matrix degradation (Galis and Khatri, 2002). According to epidemiological studies, high circulatory levels of MMP-9 are independent predictors of cardiac death and reinfarction in patients with established CAD (Blankenberg et al., 2003; Jefferis et al., 2009). It is worth noting that the levels of MMP-9 were increased in our patient cohort despite “normalized” levels of other established predictors of cardiovascular events, like CRP and IL-6.

Compared with controls, the CAD patients showed a higher 24-hour cortisol secretion and a flattened diurnal slope of cortisol. The finding of an overactivated HPA axis including raised evening cortisol, in patients with stable CAD has been confirmed in a recent study (Särndahl et al., 2010). Several other studies have indicated an association between cortisol levels and atherosclerosis although results may

seem contradictory. Dekker et al showed an independent relationship between total cortisol exposure while awake and carotid atherosclerosis (Dekker et al., 2008) while Matthews et al reported that a flattened diurnal rhythm of cortisol was related to subclinical CAD, as assessed by coronary calcification (Matthews et al., 2006). On the other hand, an increased cortisol response to acute mental stress was recently demonstrated in individuals with greater extent of coronary calcification (Hamer et al., 2010). In patients with chest pain referred for coronary angiography, the morning cortisol values based on single measurements were higher in patients with significant CAD than in patients with non-significant CAD (Bhattacharyya et al., 2008). The results obtained so far illustrate the complexity of the field and indicate that the cortisol response may differ depending on duration of disease and clinical presentation.

In the present work, a positive association between serum MMP-9 and evening salivary cortisol remained significant after the adjustment for potential confounders. The anti-inflammatory effects of glucocorticoids are assumed to include the suppression of MMP-9 activity. A number of studies have shown that glucocorticoids downregulate the expression of MMP-9 in endothelial cell lines, blood mononuclear cells and alveolar macrophages (Harkness et al., 2000; Aljada et al., 2001) and upregulate the release of TIMP-1 (Förster et al., 2007). Much less is known about the relationship between cortisol, MMP-9 and TIMP-1 levels in vivo but interestingly, one single dose of hydrocortisone given intravenously to healthy subjects has been shown to suppress proinflammatory transcription factors and reduce plasma MMP-9 within 1 hour (Aljada et al., 2001).

Normally, the release of proinflammatory cytokines will activate the HPA axis thereby providing a negative-feedback loop that inhibits the inflammatory response and restores homeostasis. It is well documented in both experimental and clinical studies that a dysregulated HPA axis is associated with enhanced inflammatory activity and development of inflammatory disease. As reported earlier by us, the high evening cortisol levels were related to systemic inflammatory activity and moreover, a suppressed cortisol response to stress was followed by an increase in CRP levels 24 hours after the stress (Nijm et al., 2007). In patients with unstable conditions of CAD, serum cortisol levels between 09.00 AM and 12.00 PM were found to be “inappropriately” low in relation to IL-6 levels (Fantidis et al., 2002). Similarly, studies on patients with rheumatoid arthritis have suggested that the release of cortisol is insufficient to inhibit on-going inflammation (Chikanza et al., 1992). However, a HPA axis failure to resolve inflammatory activity may as well reflect a state of glucocorticoid resistance in the target tissue. Underlying mechanisms of glucocorticoid resistance are not fully clarified but genetic glucocorticoid receptor variants may play a role. Interestingly, one common glucocorticoid receptor gene haplotype has been associated with a more active proinflammatory system and high cardiovascular risk (van den Akker et al., 2008). Alternative mechanisms behind the increases in MMP-9 may also involve direct cardiovascular effects of circulating cortisol. It has been shown in

experimental settings that cortisol has direct effects on the vascular wall such as remodeling, tone and inflammation (Walker, 2007) which may hypothetically contribute to raised levels of MMP-9.

The levels of MMP-9 and TIMP-1 in controls became significantly reduced after stress while the high levels of MMP-9 and TIMP-1 in CAD patients remained unchanged. One major limitation is the lack of blood samples collected at time points less than 24 hours and therefore, the data should be interpreted with great caution. It remains to be clarified to what extent the levels of MMP-9 and TIMP-1 differ between patients and controls in the early post-stress period. Nevertheless, one intriguing hypothesis is that MMP-9 and TIMP-1 are differently regulated in CAD patients and that this may be, at least partly, due to a dysfunctional HPA axis. Psychological stress is a trigger of myocardial infarction (Strike and Steptoe, 2005) and MMP-9 is, in its turn, closely associated with plaque instability. The role of MMP-9 as a possible link between stress and cardiovascular events deserves further exploration.

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**Table 1. Characteristics of CAD patients and controls.** Data are given as median (25th, 75th percentile).

	<b>Patients</b>	<b>Controls</b>	<b>p-value</b>
Age (years)	62 (56, 65)	62 (56, 66)	NS
Male/female	25/5	25/5	NS
Body mass index (kg/m <sup>2</sup> )	27 (25, 28)	28 (26, 29)	NS
Current smokers, n (%)	7 (24)	9 (31)	NS
Blood pressure, systolic (mm Hg)	138 (120, 146)	147 (131, 166)	NS
Blood pressure, diastolic (mm Hg)	80 (74, 85)	89 (78, 85)	< 0.05
White blood cell counts, cells/ $\mu$ l	6,6 (5,5, 7,5)	6,6 (5,2, 7,3)	NS
CRP, mg/ml	1,2 (0,9, 2,5)	1,6 (0,9, 3,6)	NS
IL-6, pg/ml	2,0 (1,4, 2,4)	1,8 (1,2, 2,9)	NS
24-hour cortisol secretion, nmol/l	205 (188, 256)	190 (158, 222)	< 0.05

**Table 2.** The levels of total MMP-9, active MMP-9 and TIMP-1 in CAD patients and control subjects on two consecutive days (before and after psychological stress). Data are given as median (25th, 75th percentile).

		Day 1	Day 2	Within-group change
Total MMP-9 ng/ml	<b>Patients</b>	347 (260, 426) <sup>a</sup>	358 (228, 423) <sup>b</sup>	-4.4 (-18, +15) % NS
	<b>Controls</b>	235 (196, 318)	206 (162, 259)	-19 (-57, -2) % **
Active MMP-9 ng/ml	<b>Patients</b>	85 (69, 101) <sup>a</sup>	77 (67, 98) <sup>b</sup>	0.0 (-17, +22) % NS
	<b>Controls</b>	74 (50, 84)	61 (44, 76)	-19 (-33, +3) % *
TIMP-1 ng/ml	<b>Patients</b>	169 (154, 196)	166 (148, 193)	-0.7 (-6, +3) % NS
	<b>Controls</b>	170 (157, 185)	160 (141, 184)	-7.0 (-11, +23) % **

<sup>a</sup> p < 0.05 vs controls day 1, <sup>b</sup> p < 0.01 vs controls day 2.

Differences within groups: \* p < 0.05, \*\* p < 0.01. NS; Not significant.



