FADD, Caspase-3, and Caspase-8 and Incidence of Coronary Events

Ling Xue, Yan Borné, Ingrid Yao Mattisson, Maria Wigren, Olle Melander, Marju Ohro-Melander, Eva Bengtsson, Gunilla Nordin Fredrikson, Jan Nilsson,† Gunnar Engström†

Objective—To investigate the relationship between 3 markers of apoptosis, that is, FADD (Fas-associated death domain–containing protein), caspase-3, and caspase-8, and incidence of coronary events (CEs) in a population-based cohort study.

Approach and Results—In vitro experiments were performed to assess the response of the apoptotic biomarkers after Fas stimulation of peripheral blood mononuclear cells. The experiments showed significantly increased releases of FADD, caspase-3, and caspase-8 after Fas stimulation. The relationship between FADD, caspase-3, and caspase-8, respectively, and incidence of CEs was studied in 4284 subjects from the population-based Malmö Diet and Cancer Study. Cox proportional hazards regression was used to examine the association between the apoptotic biomarkers and incidence of CE over a mean follow-up of 19 years. A total of 381 individuals had CE during the follow-up. High FADD at baseline was significantly associated with incident CE. In the highest compared with the lowest quartile of FADD, the risk factor adjusted hazards ratio for CE was 1.82 (95% confidence interval, 1.35–2.46; P for trend <0.001). A significant association was also found between caspase-8 and CE; the hazards ratio (Q4 versus Q1) was 1.90 (95% confidence interval, 1.39–2.60; P for trend <0.001) after adjustment for risk factors. No association was found between caspase-3 and CEs.

Conclusions—High levels of FADD and caspase-8, but not caspase-3, were associated with increased incidence of CE in subjects from the general population. The in vitro experiments support the view that these biomarkers could reflect activation of the extrinsic apoptotic pathway.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:983-989. DOI: 10.1161/ATVBAHA.117.308995.)

Key Words: caspase-3 • caspase-8 • coronary events • FADD

Apoptosis is a highly regulated program of cell death and can be mediated by death receptors in the plasma membrane, as well as the mitochondria and the endoplasmic reticulum.1,2 The initiation of apoptosis can be classified as intrinsic apoptotic pathway, which is initiated when facing cellular stress, and extrinsic apoptotic pathway, which is activated by extracellular ligands via cell surface death receptors.3,4 The Fas ligand binds the Fas receptor and activates the death domains at the cytoplasmic tail of the receptor. The adaptor protein FADD (Fas-associated death domain–containing protein) will then be recruited and activate caspase-8, leading to either downstream activation of the intrinsic pathway by inducing mitochondrial stress or direct activation of executioner caspases (caspase-3, caspase-6, and caspase-7) to degrade cellular components.3,5

Analyses of human atherosclerotic plaques have shown that apoptotic cell death is a common phenomenon,6 and it has been suggested that all plaque cells eventually become eliminated by apoptosis.7 Apoptotic cell death represents a possibility to remove infiltrating leukocytes and injured vascular cells without aggravating inflammation. Although this may function appropriately at early stages of the disease, increased rates of apoptosis in more advanced lesions has been associated with insufficient removal of apoptotic cells, development of secondary necrosis, and aggravation of plaque inflammation.6,8 The effect of apoptosis on atherosclerotic plaques will also depend on which cells that primarily are affected. Smooth muscle and endothelial cell apoptosis is likely to be harmful by increasing the risk of plaque rupture and thrombosis.9,10 The majority of apoptotic cells in atherosclerotic plaques are macrophages, and apoptotic macrophages have been found to localize at the site of plaque rupture in sudden coronary death.11 The role of macrophage apoptosis in atherosclerosis is more complex because it potentially can be both beneficial by reducing inflammation and harmful by inhibiting tissue repair responses orchestrated by these cells. Accordingly, experimental studies have identified both pro- and antiatherogenic effects of macrophage apoptosis in atherosclerosis.6 Transplantation of hypercholesterolemic mice with bone

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From the Department of Cardiovascular Diseases, the Second Hospital of Hebei Medical University, Shijiazhuang, China (L.X.); and Department of Clinical Sciences, Malmö, Lund University, Sweden (Y.B., I.Y.M., M.W., O.M.-M., E.B., G.N.F., J.N., G.E.).
†These authors contributed equally to this article.
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Correspondence to Ling Xue, No 215, HePing W Rd, The Second Hospital of Hebei Medical University, ShijiaZhuang, China. E-mail xueling112001@163.com; or Gunnar Engstrom, Lund University, CRC 60:13, Jan Waldenströms gata 35, 20502 Malmö, Sweden. E-mail Gunnar.Engstrom@med.lu.se
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marrow from mice with macrophages resistant to apoptosis resulted in increased plaque development.\textsuperscript{12,13} However, others have found that reduced macrophage apoptosis is associated with less plaque necrosis and lesion progression.\textsuperscript{14,15} Taken together, the available experimental and clinical data show that while apoptosis is a common phenomenon in atherosclerosis it remains to be fully understood if it contributes to an increased cardiovascular risk.

Prospective clinical studies of the association between apoptosis and risk for development of cardiovascular events have been hampered by the lack of circulating biomarkers of apoptosis. FADD, caspase-3, and caspase-8 can be measured in plasma in healthy individuals, but it is unclear to what extent they are released to blood as a result of ongoing apoptotic activity. Studies of patients with myocardial infarction have reported raised apoptotic markers in the acute phase after the event.\textsuperscript{16,17} However, to the best of our knowledge, there is no prospective study of FADD, caspase-3, or caspase-8 and the risk of developing acute coronary events (C\Es). We demonstrate here that activation of the extrinsic apoptosis pathway through the Fas receptor in human mononuclear leukocytes is associated with release of FADD, caspase-8, and caspase-3 from the cells, suggesting that the plasma levels of these factors represent potential biomarkers of apoptosis. We then analyzed baseline levels of FADD, caspase-8, and caspase-3 in 4284 subjects participating in a Swedish population-based cohort study and explored the hypothesis that increased plasma levels of these biomarkers were associated with a higher incidence of C\Es.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Activation of Apoptosis Through the Death Receptor Fas Is Associated With Release of Intracellular Components of Apoptotic Cell Signaling

Exposure of cultured human peripheral blood mononuclear cells to Fas ligand for 24 hours was associated with a dose-dependent release of FADD, caspase-3, and caspase-8 into the cell culture medium (Figure 1A). A significant increase in the extracellular release of FADD and caspase-3 was observed at a Fas ligand concentration of 2.5 \( \mu \)g/mL and continued to increase at higher concentration, while a 4-fold higher concentration of Fas ligand was required to induce secretion of caspase-8. To determine whether release of intracellular components of apoptotic cell signaling occurred also in response to factors with cytotoxic properties, we incubated cultured human peripheral blood mononuclear cells with oxidized low-density lipoprotein (LDL) in concentrations \( \leq 200 \mu \)g/mL. Although a small increase in the secretion of caspase-3 was observed at the 25 to 50 \( \mu \)g/mL of oxidized LDL, there was no induction of FADD and caspase-8 secretion (Figure 1B). Finally, we confirmed the release of FADD into the cell culture medium using Western blotting (Figure 1C). Taken together, these observations show that activation of apoptosis signaling through the Fas receptor is associated with extracellular release of FADD, caspase-8, and caspase-3 and suggests that circulating levels of these factors represent biomarkers of apoptotic activity.

Baseline Characteristics of the Study Cohort

We next investigated the association between plasma levels of FADD, caspase-8, and caspase-3 with incidence of CE in a prospective population cohort. The baseline demographic and clinical characteristics of participants in this cohort are shown in Table 1. As expected, participants with CE during follow-up had higher systolic blood pressure (BP), LDL, C-reactive protein, and lower high-density lipoprotein at baseline. In addition, individuals with CE during follow-up had higher FADD (\( P \leq 0.001 \)), caspase-8 (\( P \leq 0.001 \)), and caspase-3 (\( P = 0.046 \)) at baseline.

Correlations Between FADD, Caspase-8, and Caspase-3 and Other Risk Factors

FADD showed significant correlations with advanced age, male sex, smoking, systolic BP, LDL, and C-reactive protein; caspase-8 showed significant correlations with advanced age, male sex, systolic BP, LDL, high-density lipoprotein (inversely), C-reactive protein, education level; caspase-3 showed significant correlations with male sex, smoking, systolic BP, and LDL in the adjusted model (Table I in the online-only Data Supplement). The bivariate correlation between caspase-3 and caspase-8 was 0.542; FADD correlated significantly with caspase-3 and caspase-8 (\( r = 0.762 \) and \( r = 0.732 \), respectively). Prevalence of carotid plaque was 35%, 32%, 35%, and 32%, respectively, in the first, second, third, and fourth quartiles of FADD. The corresponding prevalence for quartiles of caspase-8 were 34%, 33%, 31%, and 35%, respectively. For quartiles of caspase-3, carotid plaque was found in 38%, 33%, 32%, and 31%, respectively. All 3 apoptotic biomarkers were inversely associated with carotid plaque after adjustments for cardiovascular risk factors (Table I in the online-only Data Supplement).

Incidence of CE in Relation to FADD, Caspase-8, and Caspase-3

During the mean follow-up of 18.9±4.5 years (80781 person-years), 381 subjects had a CE (4.72 per 1000 person-years). High FADD at baseline was significantly associated with incident CE. In the highest compared with the lowest quartile of FADD, the adjusted hazards ratio for CE was 1.82 (95% confidence interval [CI], 1.35–2.46; \( P \) for trend =0.001; Table 2 and Figure 2). This relationship remained significant also after further adjustment for carotid plaque in a sensitivity analysis.
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The C statistics value for model 2 (excluding FADD) was 0.7401 (95% CI, 0.7163–0.7638) and increased to 0.7494 (95% CI, 0.7259–0.7729) when FADD was added to the model. FADD significantly improved the discriminatory value (C statistic) for incidence of CE with 0.0093 (P = 0.006). There was no significant interaction between FADD and other cardiovascular risk factors with respect to incidence of CE. There was also no significant interaction between FADD and carotid plaque.

A significant association was also noted between caspase-8 and CE. Compared with the lowest quartile of caspase-8, the adjusted hazards ratio for CE was 1.90 (95% CI, 1.39–2.60) in the highest quartile (P for trend <0.001). The C statistics value for model 2 (excluding FADD) was 0.7401 (95% CI, 0.7163–0.7638) and increased to 0.7494 (95% CI, 0.7259–0.7729) when FADD was added to the model. FADD significantly improved the discriminatory value (C statistic) for incidence of CE with 0.0093 (P = 0.006). There was no significant interaction between FADD and other cardiovascular risk factors with respect to incidence of CE. There was also no significant interaction between FADD and carotid plaque.

The release of FADD, caspase-8, and caspase-3 into the cell culture medium was determined with ELISA. C, Western blot demonstrating a dose-dependent accumulation of a 23 kDa FADD-immunoreactive protein in PBMC stimulated with increasing concentrations of FasL.

Figure 1. Effect of apoptosis induction and toxic cell injury on cellular release of FADD (Fas-associated death domain–containing protein), caspase-8, and caspase-3. Peripheral blood mononuclear cells (PBMC) cultured in presence of 2% human serum were exposed to (A) soluble Fas Ligand (sFasL; 0.0–20.0 μg/mL) to activate apoptosis or (B) oxidized LDL (oxLDL; 0.0–200.0 μg/mL) to induce toxic cell injury. The release of FADD, caspase-8, and caspase-3 into the cell culture medium was determined with ELISA. C, Western blot demonstrating a dose-dependent accumulation of a 23 kDa FADD-immunoreactive protein in PBMC stimulated with increasing concentrations of FasL.

No association was found between caspase-3 and CE (Table 4 and Figure 4).

Discussion

Our findings demonstrate that activation of apoptosis signaling through the Fas receptor is associated with a release of the intracellular apoptosis signaling components FADD, caspase-8, and caspase-3 into the extracellular compartment, suggesting that they represent possible biomarkers of apoptotic activity. Exposure of culture mononuclear leukocytes to a cytotoxic factor, such as oxidized LDL, did not stimulate the extracellular release of FADD and caspase-8, but induced some release of caspase-3 at intermediate concentration. These observations suggest that the cellular release of FADD and caspase-8 reflects activation of apoptosis, while the release of caspase-3 may reflect both apoptosis and cell cytotoxicity in a broader sense. In a population-based cohort, we demonstrate an association between incidence of CE and high levels of FADD and caspase-8 at baseline levels, but not caspase-3. Furthermore, FADD and caspase-8 significantly improved the model discrimination in terms of C statistics, on top of several conventional risk factors for CE, and could perhaps add
information to a prediction model. The current findings supported our hypothesis that an increased rate of apoptosis, as represented by a high expression of FADD and caspase-8 in the blood, was associated with an increased incidence of CE.

Atherosclerotic vascular disease is a leading cause of myocardial infarction and heart failure, and a major factor in the development of acute coronary syndrome is disruption of an atherosclerotic plaque. It has been reported that macrophages and smooth muscle cells undergo apoptosis

Table 1. Baseline Characteristics of Participants in Malmö Diet and Cancer Study Based on Incidence of CE (n=4284)

<table>
<thead>
<tr>
<th></th>
<th>Incident CE (N=381)</th>
<th>No Incident CE (N=3903)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADD*</td>
<td>6.6 (4.7–9.7)</td>
<td>5.4 (3.8–8.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Caspase-8*</td>
<td>3.4 (2.4–4.9)</td>
<td>2.7 (2.0–3.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Caspase-3*</td>
<td>1850 (1150–2730)</td>
<td>1700 (1170–2600)</td>
<td>0.046</td>
</tr>
<tr>
<td>Men, %</td>
<td>57.5</td>
<td>42.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, y</td>
<td>59.81±5.47</td>
<td>57.24±5.93</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>88.32±12.87</td>
<td>82.76±12.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoke status</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Never smoked, %</td>
<td>32.8</td>
<td>42.1</td>
<td></td>
</tr>
<tr>
<td>Former smokers, %</td>
<td>41.2</td>
<td>36.9</td>
<td></td>
</tr>
<tr>
<td>Current smokers, %</td>
<td>26.0</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td>High alcohol consumption, %</td>
<td>3.4</td>
<td>3.4</td>
<td>0.996</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>15.5</td>
<td>6.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>148±18.94</td>
<td>140±18.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood pressure medication, %</td>
<td>23.4</td>
<td>14.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>4.37±0.94</td>
<td>4.16±0.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.27±0.35</td>
<td>1.41±0.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lipid-lowering medication, %</td>
<td>2.9</td>
<td>1.8</td>
<td>0.147</td>
</tr>
<tr>
<td>CRP,* mg/L</td>
<td>1.6 (0.9–3.5)</td>
<td>1.3 (0.6–2.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Education level</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High education, %</td>
<td>57.2</td>
<td>44.4</td>
<td></td>
</tr>
<tr>
<td>Medium education, %</td>
<td>32.0</td>
<td>35.9</td>
<td></td>
</tr>
<tr>
<td>Low education, %</td>
<td>10.8</td>
<td>19.7</td>
<td></td>
</tr>
<tr>
<td>Carotid plaque, %</td>
<td>50.1</td>
<td>32.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

CE indicates coronary event; CRP, C-reactive protein; FADD, Fas-associated death domain–containing protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; and SBP, systolic blood pressure.

*FADD, caspase-8, caspase-3, and CRP are presented as median (interquartile range in brackets) because of skewed distributions, and log-transformed values are used to calculate P values. All other values are means±SD, unless otherwise stated.

Table 2. Incidence and Hazard Ratios of CE in Relation to Quartiles of FADD (n=4284)

<table>
<thead>
<tr>
<th>FADD</th>
<th>Q1 (n=1071)</th>
<th>Q2 (n=1071)</th>
<th>Q3 (n=1071)</th>
<th>Q4 (n=1071)</th>
<th>P(Trend)</th>
<th>HR per 1 SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FADD, AU</td>
<td>1.29–3.86</td>
<td>3.86–5.50</td>
<td>5.50–8.10</td>
<td>8.10–58.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men/women</td>
<td>360/711</td>
<td>398/673</td>
<td>438/633</td>
<td>481/590</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE per 1000 (men/ women)†</td>
<td>4.7/2.3</td>
<td>5.6/2.1</td>
<td>8.6/3.7</td>
<td>9.5/5.2</td>
<td>&lt;0.001</td>
<td>1.30 (1.18–1.44)</td>
</tr>
<tr>
<td>Model 1</td>
<td>1.00</td>
<td>1.11 (0.79–1.56)</td>
<td>1.70 (1.25–2.31)</td>
<td>2.05 (1.52–2.78)</td>
<td>&lt;0.001</td>
<td>1.30 (1.18–1.44)</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.00</td>
<td>1.08 (0.77–1.51)</td>
<td>1.55 (1.14–2.11)</td>
<td>1.82 (1.35–2.46)</td>
<td>&lt;0.001</td>
<td>1.25 (1.13–1.38)</td>
</tr>
</tbody>
</table>

Model 1: Adjusted for age and sex. Model 2: adjusted for age, sex, waist circumference, smoking (current, former, and never), alcohol consumption, diabetes mellitus, systolic blood pressure, BP-lowering drug, LDL, HDL, lipid-lowering drug, education level (3 groups), and C-reactive protein. AU indicates arbitrary units; BP, blood pressure; CE, coronary event; FADD, Fas-associated death domain–containing protein; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

*HR per 1 standard deviation increment of log-transformed biomarker.
†Incidence of coronary events per 1000 person-years in men/women.
in unstable atherosclerotic plaques, which can lead to rupture of the plaque and thrombosis, and finally cause acute myocardial infarction. However, the role of apoptosis in atherosclerosis remains a matter of controversy because it potentially can have both beneficial and pathogenic effects. Apoptotic cell death in lesions is probably less harmful than necrotic cell death, which initiates inflammation and damage to neighboring cells. In contrast, apoptosis is an energy requiring form of programmed cell death, whereby damaged cells are removed without provoking inflammation. The importance of this for atherosclerosis is exemplified by studies demonstrating that impaired removal of apoptotic cells in atherosclerotic plaques is associated with a progression of apoptotic cells into secondary necrosis, enhanced inflammation, and necrotic core expansion. Moreover, Gautier et al reported that increased macrophage apoptosis is associated with reduced atherosclerosis during early stages of the disease but aggravates atherosclerosis at more advanced stages in cholesterol-fed apoE−/− mice. Taken together, the available experimental data suggest that the concentration of apoptotic biomarkers is not mainly a question of atherosclerotic risk factors or atherosclerotic burden. Because apoptosis occurs in many tissues and cell types, the circulating apoptotic biomarkers may be of a nonvascular origin. However, experimental studies have identified both pro- and antiatherogenic effects of macrophage apoptosis in atherosclerosis, and increased plaque development has been found in hypercholesterolemic mice that were treated with bone marrow from mice with macrophages resistant to apoptosis. Apoptotic macrophages have also been found to localize at the site of plaque rupture in sudden coronary death, which suggest that apoptosis could be involved in plaque rupture. To what extent the plasma levels of FADD and caspase-8 reflect the activity of disease within lesions requires further investigation.

Even though all 3 apoptotic biomarkers were strongly correlated (all \( r > 0.5 \)), caspase-3 was not significantly associated with CE. It is known that caspase-8 can activate procaspase-3, which in turn can cleave and activate caspases 6 and 7, leading to characteristic cell changes and death. The nonsignificant relationship between caspase-3 and incidence of CE could reflect the fact that apoptosis is a highly regulated and controlled process, and there may be other factors that inhibit or activate the apoptotic cascade. However, our experiments showed that caspase-3 may be released both from apoptosis and from cell cytotoxicity. Caspase-3 could, therefore, be less specific marker of apoptosis.

**Strengths and Limitations**

This study was based on a large number of subjects, a long follow-up period, and a large number of incident CE. CE
included fatal or nonfatal myocardial infarction from the hospital discharge register and death because of ischemic heart disease outside the hospital. Data from the Hospital Discharge register have shown high case validity in previous studies.28

The participation rate was ≈41% at the baseline examination in 1992 to 1994. One question is whether the cohort is representative for the general population. It has been shown that the overall mortality rates were higher in nonparticipants,29 which is in line with most cohort studies. However, prevalence of smoking, obesity, and education level was largely similar in participants of Malmö Diet and Cancer study compared with the results from a mailed questionnaire from the same city, with a participation rate of 75%.29 It is reasonable to think that the results could be generalized to the general adult population in Sweden.

Some risk factors could have changed over the follow-up period. For example, it is well known that many individuals in Sweden have quit smoking in the past decades. However, if anything, this would reduce the risk of CE in those who smoked at baseline and bias the results toward null.

Conclusion
Our results suggest that high levels of FADD and caspase-8 represent biomarkers of apoptosis and that elevated plasma levels of these markers are associated with increased incidence of CE. This relationship is independent of several potential confounding factors. FADD and caspase-8 significantly increased the model discrimination in terms of C statistics. Further research is required to confirm these findings and to explore the underlying mechanisms between raised apoptotic biomarkers and incidence of CE.

Acknowledgments
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### Table 4. Incidence and Hazard Ratios of CE in Relation to Quartiles of Caspase-3 (n=4284)

<table>
<thead>
<tr>
<th>Caspase-3</th>
<th>Q1 (n=1071)</th>
<th>Q2 (n=1071)</th>
<th>Q3 (n=1071)</th>
<th>Q4 (n=1071)</th>
<th>P(Trend)</th>
<th>HR per 1 SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3, AU</td>
<td>94.9–1076</td>
<td>1077–1707</td>
<td>1707–2630</td>
<td>2630–11971</td>
<td>0.14</td>
<td>1.10 (0.99–1.21)</td>
</tr>
<tr>
<td>Men/women</td>
<td>382/689</td>
<td>393/678</td>
<td>433/638</td>
<td>469/602</td>
<td>0.71</td>
<td>1.03 (0.93–1.14)</td>
</tr>
<tr>
<td>CE per 1000† (men/women)</td>
<td>6.5/2.8</td>
<td>7.6/2.8</td>
<td>6.7/4.0</td>
<td>8.0/3.4</td>
<td>0.14</td>
<td>1.10 (0.99–1.21)</td>
</tr>
</tbody>
</table>

Model 1: Adjusted for age and sex.
Model 2: Adjusted for age, sex, waist circumference, smoking (current, former, and never), alcohol consumption, diabetes mellitus, systolic blood pressure, BP-lowering drug, LDL, HDL, lipid-lowering drug, education level (3 groups), and CRP. AU indicates arbitrary units; BP, blood pressure; CE, coronary event; CRP, C-reactive protein; FADD, Fas-associated death domain–containing protein; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

*HR per 1 standard deviation increment of log-transformed biomarker.
†number of coronary events per 1000 person-years in men/women.
Disclosures

None.

References


Highlights

- FADD (Fas-associated death domain–containing protein), caspase-8, and caspase-3 are released after Fas stimulation of human peripheral blood mononuclear cells, suggesting that these biomarkers could reflect activation of the extrinsic apoptotic pathway.
- High levels of FADD and caspase-8, but not caspase-3, were associated with increased incidence of coronary events in subjects from the general population.
- FADD and caspase-8 improved the model discrimination in terms of C statistics, on top of several conventional cardiovascular risk factors.
- The results support the hypothesis that an increased rate of apoptosis, as represented by a high expression of FADD and caspase-8 in the blood, is associated with an increased incidence of CE.
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Material and Methods

In vitro response of FADD from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC’s) were isolated from healthy blood donors and seeded at a density of 0.5x10^6 cells in a 96-well plate in cRPMI and in presence of 2% human serum (Sigma Aldrich). Soluble FAS ligand (0-20 µg/ml) and copper oxidized LDL (0-200 µg/ml) were used for treatment of cells for 24h, 37°C with 5% CO₂. Medium from the cells were subsequently analyzed with human protein FADD ELISA kit (MyBioSource, San Diego, CA, USA), Casp-3 colorimetric assay (Abcam) and Casp-8 ELISA (Abcam). For western blotting PBMC were seeded and stimulated 24h with 0, 5 and 20 µg/ml soluble Fas ligand as described above. After stimulation, medium from each well was collected and frozen in -20°C. Protein was precipitated in 95% EtOH in -20°C o/n and concentration was determined using Pierce BCA protein concentration (Thermo Scientific, IL, USA). Subsequently, 88 µg of each sample was dissolved in 6x SDS loading buffer (Bio-Rad) and 6 µl β-mercaptoethanol and loaded to a 4-20% Mini-PROTEAN TGX protein gel (Bio-Rad, CA, USA). The gel was run in 1x Tris/Glycine/SDS buffer (Bio-Rad) at 100V for 10 min followed by 200V for 30 min. Proteins were then transferred to a PVDF-membrane (Bio-Rad) pre-wetted with 100% MeOH and transferred in a wet sandwich blot 100V, 60 min in 4°C followed by blocking in 5% milk 1x S&B in 4°C o/n. Membrane was then incubated 1hr with monoclonal rabbit anti-FADD (Abcam, Cambridge, UK) 2000x diluted in 5% milk 1xTBS-Tween, followed by incubation 1hr with HRP goat anti-rabbit (Dakocytomation, Glostrup, Denmark) diluted 2000x in 5% milk 1xS&B. ChemiGlow West Chemiluminescence Substrate Kit (Protein Simple, CA, USA) was used according to manufacturer’s instructions for detection using a Licor imager (Bio-Rad).
Study Population

This study used the cardiovascular cohort of the Malmö Diet and Cancer Study (MDCS-CC)\(^1\), which is a random sample of the Malmö Diet and Cancer Study (MDCS)\(^1,2\). MDCS is a cohort study from the general population in Malmö. All individuals in the city born between 1923-1945 (men) and 1923-1950 (women) were invited through letters or advertisements in the press. Participant rate was approximately 41%. The cohort characteristics, data collection methods and inclusion criteria for MDCS have been described elsewhere\(^1-3\). A total of 28,449 individuals (men =11,246, women =17,203) underwent a baseline examination between March 1991 and September 1996. Samples of peripheral venous blood, blood pressure measurements and anthropometric measures were taken and participants filled out self-administered questionnaires. Between October 1991 and February 1994, a group of 6103 individuals was randomly selected to be recruited in MDCS-CC, which originally was designed to study the epidemiology of carotid artery disease\(^3\). There were 5540 participants who came to the second visit for collecting fasting plasma. After excluding 368 subjects due to incomplete clinical data and another 307 subjects with missing plasma samples, the remaining 4865 samples were sent for analysis. Furthermore, after excluding samples not passing the internal quality control for the biomarker analysis, the cohort included 4601 individuals with data available on FADD, 4529 with caspase-3 and 4643 with caspase-8. There were 4467 individuals with complete data on FADD, caspase-8 and caspase-3. After excluding participants with history of coronary events (CE) (n=76) and missing values for covariates (n=107), the final cohort consisted of 4284 subjects. All participants provided written informed consent. The study was approved by the Regional Ethical Review Board in Lund, Sweden (LU 51/90) and was carried out in accordance with the Helsinki Declaration.
Measurements and Definitions

Information on the current use of blood pressure-lowering and lipid-lowering medications, smoking habits, alcohol consumption and education level were gathered through self-administered questionnaires. Subjects were grouped into current smokers (smoke regularly or occasionally), former smokers and never-smokers. High alcohol consumption was defined as > 40 g alcohol per day for men and > 30 g per day for women. High education was defined as >12 study years, medium education as 9-12 study years whereas low education was defined as <9 study years. Waist circumference was measured midway between the lowest rib margin and iliac crest in centimeters. Blood pressure (BP) was measured using a mercury column sphygmomanometer after 10 minutes of rest in the supine position. Fasting blood glucose, total cholesterol and high density lipoprotein (HDL) levels were measured from fresh blood samples according to standard procedures at the Department of Clinical Chemistry, Malmö University Hospital. Low density lipoprotein (LDL) concentrations were determined according to Friedewald’s formula. C-reactive protein (CRP) was analysed using the Tina-quant CRP latex assay (Roche Diagnostics, Basel, Switzerland) on an ADVIA® 1650 Chemistry System (Bayer Healthcare, NY, USA).

Participants underwent B-mode ultrasonography of the right carotid artery by trained certified sonographers, using an Acuson 128 (Acuson, Mountain View, California). In short, the bifurcation area of the right common carotid artery was scanned within a predefined window comprising 3 cm of the right common carotid artery (CCA), the bifurcation, and 1 cm of both the internal and external carotid artery for the presence of plaque. Carotid plaque was defined as a focal thickening of the IMT >1.2 mm and a minimum area of 10 mm². Information about carotid plaque was missing for 166 individuals.

Caspase-8 levels were measured using Proseek® Multiplex CVD 196×96 reagent kit, based on PEA, a Proximity Extension Assay technology. FADD and Caspase-3 were measured using...
Proseek® Multiplex Oncology I96×96. High throughput real-time qPCR was performed by using the Fluidigm® BiomarkTM or Fluidigm® BiomarkTM HD systems. The data was analysed using a pre-processing normalization procedure. In the analysis, the value for extension control was subtracted to get delta Cq (dCq) values in order to normalize each sample. Then, normalization between samples was done by subtracting the interplate control for each assay. In this way, the values were set relative to a fixed level.

FADD, Caspase-8 and Caspase-3 are expressed in arbitrary units which can be used for statistical multivariate analysis and represents relative quantification between samples in arbitrary units (OLINK Bioscience, 2013. www.olink.se). The measuring range (i.e., lower and upper limits of quantification) was 7.8 ng/ml to 1000 ng/ml for FADD, 3.8 to 62500 pg/ml for Caspase-8 and 1.9 to 31250 pg/ml for Caspase-3. The coefficient of variation (CV) for FADD was 13% based on values over the limit of detection (LOD: 7.8 ng/ml), 22% for Caspase-8 (LOD: 0.48 pg/ml), and 16% for Caspase-3 (LOD: 1.91 pg/ml).

For all statistical analyses, the apoptotic biomarkers are categorized into quartiles, or log-transformed values of the apoptotic biomarkers are used.

Follow-up and end-point retrieval

The time variable was calculated for all subjects as the calendar time (in years) from the baseline examination in 1992-1994 until first CE, death, emigration from Sweden, or until December 31st, 2013, whichever came first. Ascertainment of cases was through the Swedish Hospital Discharge Register and the National Cause of Death Register. The validity of the registries has been proven to be high. A CE was defined as a fatal or non-fatal myocardial infarction (International Classification of Diseases 9th and 10th revisions (ICD-9 and ICD-10) codes 410 and I21, respectively) or death due to ischemic heart disease (codes 411, 412 and 414 (ICD-9) or I22-I25 (ICD-10)).
Statistical Analysis

FADD, Caspase-3, Caspase-8 and CRP showed a right-skewed distribution and were logarithmically transformed. Their medians with corresponding interquartile range are presented. Linear regression was used to assess the relationships between FADD, Caspase-8 and Caspase-3 and other risk factors. FADD, Caspase-8 and Caspase-3 were classified into quartiles. Cox’ proportional hazards regression was used to examine the association between FADD, Caspase-8 or Caspase-3 and incidence of CE. The lowest quartile was used as the reference category. Hazard ratios (HR) with 95% confidence interval were calculated. Age and sex were included as covariates in the model 1. In the full multivariable model 2, we also adjusted for cardiovascular risk factors, i.e., waist circumference, smoking, alcohol consumption, diabetes, systolic blood pressure (SBP), blood pressure (BP)-lowering drug, LDL, HDL, lipid-lowering drug, CRP and education level, in addition to age and sex. Age, waist circumference, systolic blood pressure, LDL, HDL and CRP were fitted as continuous variables, and sex, diabetes, alcohol, BP-lowering drug and lipid-lowering drug were used as dichotomous. Three categories were used for education and smoking. Since carotid plaque can be considered as an intermediary link in the causal chain between biomarkers and coronary events, adjustments for carotid plaque (yes vs no) was performed in a sensitivity analysis only. The appropriateness of the Cox’ proportional hazards model was assessed visually by plotting the log minus log-plots, i.e., the cumulative survival estimate after the ln(-ln) transformation. Possible effect modification of age, sex, smoking, LDL, systolic blood pressure and diabetes, with respect to the association between FADD, Caspase-8 or Caspase-3 and incident CE, were investigated by introducing interaction terms in the multivariate model. Kaplan-Meier plots were used to illustrate the quartiles of FADD, Caspase-8 or Caspase-3 in relation to incidence of CE. Model discrimination was estimated with Harrell’s C-statistics\textsuperscript{6}. 
A p-value < 0.05 was considered significant. IBM SPSS Statistics 22 (Chicago, Illinois, USA) and Stata software version 12.0 (Stata Corp, College Station, TX, USA) were used for the statistical analysis.
References


Supplemental Table

Supplemental Table I. Relationships between FADD, caspase-8 and caspase-3 and other risk factors.

<table>
<thead>
<tr>
<th></th>
<th>FADD</th>
<th>Caspase-8</th>
<th>Caspase-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model 1</td>
<td>Model 2</td>
<td>Model 1</td>
</tr>
<tr>
<td>Sex (female vs male)</td>
<td>-0.089***</td>
<td>-0.084***</td>
<td>-0.139***</td>
</tr>
<tr>
<td>Age</td>
<td>0.085**</td>
<td>0.067***</td>
<td>0.131***</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>-0.093***</td>
<td>0.001</td>
<td>0.170**</td>
</tr>
<tr>
<td>Current smoking (yes vs no)</td>
<td>0.047**</td>
<td>0.050**</td>
<td>0.016</td>
</tr>
<tr>
<td>High Alcohol Consumption (yes vs no)</td>
<td>0.021</td>
<td>0.010</td>
<td>0.030</td>
</tr>
<tr>
<td>Diabetes (yes vs no)</td>
<td>0.025</td>
<td>-0.007</td>
<td>0.058***</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>0.111***</td>
<td>0.075***</td>
<td>0.139***</td>
</tr>
<tr>
<td>Blood pressure medications (yes vs no)</td>
<td>0.037*</td>
<td>0.008</td>
<td>0.061***</td>
</tr>
<tr>
<td>LDL</td>
<td>0.062***</td>
<td>0.044**</td>
<td>0.091***</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.065***</td>
<td>-0.018</td>
<td>-0.124***</td>
</tr>
<tr>
<td>Lipid medications (yes vs no)</td>
<td>-0.004</td>
<td>-0.002</td>
<td>-0.008</td>
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<tr>
<td>CRP</td>
<td>0.082***</td>
<td>0.051**</td>
<td>0.128***</td>
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<tr>
<td>Low education level (yes vs no)</td>
<td>0.038*</td>
<td>0.010</td>
<td>0.068***</td>
</tr>
<tr>
<td>Carotid plaque (yes vs no)</td>
<td>-0.016</td>
<td>-0.064***</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Values are standardized beta coefficients from a linear regression. Log transformed values were used for CRP, FADD, Caspase-8 and Caspase-3.

Model 1: crude.

Model 2: adjusted for age, sex, waist circumference, smoking, alcohol consumption, diabetes, systolic blood pressure, BP-lowering drug, LDL, HDL, Lipid-lowering drug, education level and CRP.

*p<0.05 **p<0.01 ***p<0.001.