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Inflammatory cells, ceramides, and expression of proteases in perivascular adipose tissue adjacent to human abdominal aortic aneurysms

Maggie Folkesson, PhD,‡ Emina Vorkapic, MSc,‡ Erich Gulbins, MD,§ Lukasz Japtok, PhD,‡ Burkhard Kleuser, PhD,‡ Martin Welander, MD,§ Toste Länne, MD,§ and Dick Wågsäter, PhD,‡

Background: Abdominal aortic aneurysm (AAA) is a deadly irreversible weakening and distension of the abdominal aortic wall. The pathogenesis of AAA remains poorly understood. Investigation into the physical and molecular characteristics of perivascular adipose tissue (PVAT) adjacent to AAA has not been done before and is the purpose of this study.

Methods and Results: Human aortae, periaortic PVAT, and fat surrounding peripheral arteries were collected from patients undergoing elective surgical repair of AAA. Control aortas were obtained from recently deceased healthy organ donors with no known arterial disease. Aorta and PVAT was found in AAA to larger extent compared with control aortas. Immunohistochemistry revealed neutrophils, macrophages, mast cells, and T-cells surrounding necrotic adipocytes. Gene expression analysis showed that neutrophils, mast cells, and T-cells were found to be increased in PVAT compared with AAA as well as cathepsin K and S. The concentration of ceramides in PVAT was determined using mass spectrometry and correlated with content of T-cells in the PVAT.

Conclusions: Our results suggest a role for abnormal necrotic, inflamed, proteolytic adipose tissue to the adjacent aneurysmal aortic wall in ongoing vascular damage. (J Vasc Surg 2016;●:1-9.)

Clinical Relevance: Abdominal aortic aneurysm is an inflammatory disease. This study shows that adipocytes surrounding the aorta may be a great source of inflammatory leukocytes that are attracted by adipocytes undergoing necrosis and by proinflammatory ceramides. Future strategies preventing the formation of perivascular adipose tissue and targeting inflammation from the adventitial side must be taken into consideration.

The prevalence of abdominal aortic aneurysm (AAA) affects around 2% of the world’s population and has declined from higher numbers mainly because of a decreased smoking rate. Unfortunately, there are still no treatments for this disease other than invasive and costly procedures to replace or reinforce the dilated aortic segment. The mechanism of pathogenesis of AAA is not entirely clear yet. Thus, in order to understand this disease better, a closer investigation of AAA and its surrounding tissue is necessary.

AAA has been classified as a vascular matrix degenerative disease that causes an enlargement to the abdominal segment of the aorta. Various proteases capable of degrading the matrix proteins in the aorta have been found in the abdominal aorta and its intraluminal thrombus. Previous studies depict leukocytes as sources for production of proteases capable of degrading the aortic wall, thus, enlargement of the AAA.

In all tissues, recruitment of immune cells or inflammation begins at postcapillary venules. Human abdominal aorta lacks vasa vasmor. Facilitated immune cell recruitment, however, occurs through neovascularization that is frequently seen in adventitia layer of aneurysmal aorta. Adjacent to the adventitia layer of the aorta, a supporting tissue of fat depot comprising of a mixture of white and brown fat shields the vessel against the surrounding tissues. This perivascular adipose tissue (PVAT) varies in amount and content in different disorders and is highly vascularized. PVAT regulates vascular function by producing a large number of vasocrine molecules. Excessive accumulation of inflamed, dysfunctional PVAT has been proposed to be a major risk factor for endothelial dysfunction and atherosclerosis.
Abdominal obesity is a risk factor for AAA and also correlates to the quantity of PVAT. Further, the quantity of PVAT could be examined clinically by computer tomography and is associated with aneurysmal diameter. A growing body of evidence indicates that adipose tissue functions more than just a storage organ. Adipose tissue is a dynamic participant in normal physiology and in the development of several diseases. In particular, PVAT secretes cytokines and other factors that contribute to sterile inflammation of vessels in atherosclerosis. PVAT has been shown to be responsible for production of adipokines such as interleukin (IL)-6, which is elevated in the plasma of AAA patients. Surprisingly, the release of these factors by PVAT has not been investigated in AAA.

Sphingolipid metabolites, particularly ceramide and sphingosine-1-phosphate (SIP), are signaling molecules that regulate a diverse range of cellular processes that are important in immunity, inflammation, and inflammatory disorders. It has previously been shown that adipose tissue is infiltrated with macrophages and is a source of ceramides that can induce inflammation. Ceramides are fatty acids of various lengths, and their synthesis is stimulated by long-chain fatty acids that are readily found in adipocytes. Mammalian cells generally have ceramides with backbone of C16 to C26 carbons. Evidence suggests that adipose tissue inflammation and abnormalities in sphingolipid metabolism may contribute to the metabolic disorders associated with obesity, and it has been shown that ceramide is implicated in the pathogenesis of obesity and cardiovascular disease. Ceramides regulate apoptosis and necrosis; they activate protein kinases and are capable of inducing cell-signaling cascades. Cytokines such as tumor necrosis factor (TNF)-α induce inflammation through ceramides and matrix metalloproteinase (MMP) participate in production and activation of ceramides.

The purpose of this study is to elucidate the role of PVAT in the pathogenesis of AAA by investigating the composition of PVAT in AAA.

METHODS

Sample collection. Patients undergoing elective surgery at Linköping University Hospital, demonstrating an aortic diameter >55 mm with computed tomography scan, were included in the study. No inflammatory or mycotic aneurysm was included. Patients characteristics are described in the Supplementary Table (online only). The aneurysmal tissue from the anterior excess sac was removed in the safest way from 19 patients, and peripheral arteries of the abdominal wall were taken during the opening from four patients. Aneurysmal biopsies from patients were gently divided by surgical adventicectomy under a light microscope into following four parts: (1) intima/media; (2) adventitia; (3) whole AAA wall without PVAT; and (4) PVAT surrounding the AAA vessel.

Control infrarenal aortas were obtained from nine human controls and nine aneurysmal tissues including the PVAT were homogenized with TissueLyser using safe-lock tubes with a metal bead and trizol-chloroform. Total RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany) and reversely transcribed with random primers and Superscript III (Invitrogen, Carlsbad, Calif) according to manufacturer’s instructions. Quantitative real-time polymerase chain reaction (PCR) was performed on a 7500 Fast Real-time PCR Sequence
Detector (Applied Biosystems, Foster City, Calif) using gene expression assay primers and TaqMan Universal Fast PCR Mastermix (Applied Biosystems) with the following cycling conditions: 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds. Samples were run in duplicate and semiquantified against a standard curve. Results were normalized to human control gene Tbp.

**Mass spectrometry.** PVAT from AAA (n = 12) and from peripheral arteries of the abdominal wall from AAA patients (n = 4) were collected from the same cohort for analysis of sphingolipid metabolites. Ceramides, sphingosine (SPH), and S1P, were extracted and quantified as recently described.25 To the samples, water was added; the samples were homogenized using a tip sonicator. Briefly, lipid extraction of tissue fat samples was performed using C17-ceramide, C17-SPH, and C17-S1P as internal standards. Sample analysis was carried out by rapid-resolution liquid chromatography-tandem mass spectrometry using a quadrupole time-of-flight (QTOF) 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive electrospray-ionization mode. The precursor ions of SPH (m/z 300.289), C17-SPH (m/z 286.274), S1P (m/z 380.256), and C17-S1P (m/z 366.240) were cleaved into the fragment ions of m/z 282.280, m/z 268.264, m/z 264.270, and m/z 250.252 respectively. Ceramides (C16-ceramide [m/z 520.508], C17-ceramide [m/z 534.524], C16-ceramide [m/z 548.540], C18:1-ceramide [m/z 547.524], C20-ceramide [m/z 546.540]), C22-ceramide [m/z 604.602], C24-ceramide [m/z 632.634], and C24:1-ceramide [m/z 630.618]) were cleaved into the fragment ion of m/z 264.270. Quantification was performed with Mass Hunter Software (Agilent Technologies).

**Statistical analysis.** The statistical analysis was performed with IBM SPSS Statistics v 22 (IBM, Chicago, Ill). All measurements are represented as median and interquartile range, and P values of <.05 are considered statistically significant. Two-group comparisons (PVAT vs control aorta, adventitia and intima/media layer of AAA) of the quantitative data were performed using the nonparametric Mann-Whitney rank sum test. Pearson correlation coefficient was calculated to analyze the statistical correlation between ceramide metabolites and leukocyte markers.

**RESULTS**

AAA is surrounded by abundant PVAT. Fig 1, A shows a common encounter of vascular surgeons after exposing the enlarged aorta during vascular repair surgery of AAA. As it can be seen in this figure, AAA is surrounded by PVAT. In agreement with human AAA, presence of substantial amount of PVAT can also be seen in angiotensin II-induced aneurysmal model of AAA (Fig 1, B), something that is not seen in control aortas in the same amount (Fig 1, C).

PVAT surrounding AAA contains necrotic adipocytes and a sterile inflammatory infiltrate. Perilipin is a lipid droplet that surrounds adipocytes. Lack of this protein in fat cells indicates necrosis. We have identified necrotic (perilipin negative) adipocytes in adventitia and PVAT of AAA. Fig 2, A-B shows a serial section staining experiment of AAA containing PVAT where presence of necrotic adipocytes is seen. These necrotic cells appear to be surrounded by neutrophils (CD66b positive staining) shown in Fig 2, C and macrophages (CD68 positive staining) shown in Fig 2, D.

Presence of several other inflammatory cells and proteases were also observed in PVAT of AAA patients. In Fig 3, A, we show presence of tryptase positive cells, a marker for mast cells, and Fig 3, B shows presence of CD3 positive cells, a marker of T-cells. Fig 3, C shows presence of MMP-9, the most studied protease in regard to pathogenesis of AAA. Besides the PVAT, presence of inflammatory cells in the AAA is usually seen in media and adventitia layer of aorta (data not shown). Nonaneurysmal control aortas have very
few adipocytes in general and no necrotic adipocytes have been found in this study (data not shown). Protein data was confirmed by gene expression analysis. To start with, we analyzed messenger RNA mainly expressed by adipocytes to validate integrity of our PVAT. We found that perilipin was expressed in greater extent in PVAT as expected compared with all layers of aneurysmal aortas as well as non-aneurysmal control aortas (Fig 4, A). In line with this result, adiponectin, a protein expressed by adipocytes, showed to be expressed largely by PVAT compared with other samples (Fig 4, B).

To evaluate vascular infiltration of inflammatory cells within the different layers of the vessel wall as well as within PVAT, gene expression analysis was performed on markers for immune cells commonly studied with respect to AAA. Gene expression of the neutrophil marker, CD66b, was greater in PVAT compared with adventitial layer of the aneurysmal aorta as well as control aortas ($P < .05$; Fig 4, F).

Fig 2. Immunohistochemistry. A, Perilipin; B, enlarged perilipin; C, CD66b; and D, CD68. The arrows show 3,3'-diaminobenzidine (DAB) brown positive staining in each image except in (B), which shows perilipin negative necrotic fat cells.

Fig 3. Presence of mast cell tryptase (A), T-cells (B), and matrix metalloproteinase (MMP)-9 (C) in perivascular adipose tissue (PVAT; arrows).
Gene expression of the macrophage marker CD68 was equally expressed in PVAT compared with normal aorta and adventitia layer of AAA samples (Fig 4, C). Macrophages were predominantly found in the intima/media layer of the aneurysmal vessel otherwise. Gene expression analysis demonstrated a three-fold increase of the mast cell marker mast cell chymase in PVAT compared with the intima/media layer of aneurysmal aortas (P < .01) and compared with nonaneurysmal control aortas (P < .01; Fig 4, E). The expression of T-cell marker CD3e was significantly increased in PVAT compared with nonaneurysmal control aortas (P < .05) and in levels equal to AAA (Fig 4, D).

In other words, neutrophils and mast cells were the only cell markers that showed an expression to a higher extent in PVAT compared with aneurysmal wall. Nevertheless, markers for macrophages and T-cells were also detected. These results are in line with immunohistochemical observations with over presentation of mast cells and neutrophils in PVAT, (Fig 3, A-B).

**PVAT as a possible source of inflammatory cytokines and proteases.** We further analyzed the expression level of several cytokines and proteases in PVAT that have been shown to participate in pathogenesis of AAA. IL-6 was found to be increased in PVAT compared with intima/media of aneurysmal aortas by four-fold (P < .05) (Fig 5, A). Cell death is another hallmark of AAA. Gene expression of caspase 3, a marker of apoptosis, was increased in the aneurysmal aorta by 30% (P < .05) compared with nonaneurysmal control aortas but detected at equal levels in PVAT compared with AAA samples (Fig 5, B).
To elucidate the expression of proteases, cathepsin K, L, and S were examined. All three cathepsins were highly expressed in intima/media of aneurysmal aortas (Fig 5, C-E). In PVAT, the mRNA expression of cathepsin K and cathepsin S were also found to be increased by 47% ($P < .05$) and by 116% ($P < .05$) compared with nonaneurysmal control aortas.

To establish a broader protease profile of other significant mediators of AAA, gene expression analyses were also performed on the metalloproteinases MMP-2, MMP-8, MMP-9, and MMP-12 but also on the proinflammatory cytokine TNF-$\alpha$ (Supplementary Fig, online only).

Sphingolipid metabolite profile in PVAT possible responsible for infiltration of T-cells in AAA. We considered whether sphingolipid metabolites such as ceramides could be one of the driving proinflammatory factors associated with leukocyte infiltration in PVAT and AAA and analyzed the ceramide content as well as some of the target molecules important in the biogenesis of ceramides in PVAT from AAA and peripheral arteries from AAA patients (Table I). When correlating these lipids with gene expression of markers for leukocytes in PVAT, interesting results were observed (Table II). Markers of macrophages, mast cells, and neutrophils were, in general, negatively associated with the lipids with few exceptions. Specifically, macrophages where strongly negatively associated with C16-ceramides in PVAT ($r = -0.81; P = .05$; Fig 6, A), whereas neutrophils were negatively associated with C24:1-ceramides in adventitia of AAA aortas ($r = -0.81; P < .05$; Fig 6, B) but positively with S1P in PVAT ($r = 0.82; P < .05$; Fig 6, C). In contrast, markers for T-cells were in general positively associated with the most of the molecules in both PVAT and adventitia layer of the aorta reaching a significant correlation in adventitia with C16-ceramide of PVAT ($r = 0.91; P < .05$; Fig 6, D).
Table I. Sphingolipid content in perivascular adipose tissue (PVAT) and peripheral fat from patients with abdominal aortic aneurysm (AAA)

<table>
<thead>
<tr>
<th></th>
<th>PVAT (n = 12)</th>
<th>Peripheral fat (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer16, pmol/mg protein</td>
<td>879 ± 947</td>
<td>1435 ± 785</td>
</tr>
<tr>
<td>Cer18, pmol/mg protein</td>
<td>228 ± 360</td>
<td>320 ± 103</td>
</tr>
<tr>
<td>Cer20, pmol/mg protein</td>
<td>88 ± 88</td>
<td>173 ± 28</td>
</tr>
<tr>
<td>Cer22, pmol/mg protein</td>
<td>316 ± 329</td>
<td>588 ± 179</td>
</tr>
<tr>
<td>Cer24, pmol/mg protein</td>
<td>455 ± 434</td>
<td>584 ± 175</td>
</tr>
<tr>
<td>Cer24:1, pmol/mg protein</td>
<td>850 ± 722</td>
<td>1296 ± 414</td>
</tr>
<tr>
<td>Total ceramides, pmol/mg protein</td>
<td>2816 ± 2832</td>
<td>4397 ± 464</td>
</tr>
<tr>
<td>Sphingosine, pmol/mg protein</td>
<td>69 ± 42</td>
<td>80 ± 27</td>
</tr>
<tr>
<td>S1P, pmol/mg protein</td>
<td>2.2 ± 6.4</td>
<td>1.1 ± 1.0</td>
</tr>
<tr>
<td>ASM, pmol/h/mg protein</td>
<td>11.6 ± 7.7</td>
<td>6.7 ± 1.2</td>
</tr>
</tbody>
</table>

ASM, Acid sphingomyelinase; S1P, sphingosine-1-phosphate.

Table II. Correlation (r value) between sphingolipid content and leukocyte markers in perivascular adipose tissue (PVAT)

<table>
<thead>
<tr>
<th></th>
<th>Macrophages</th>
<th>Neutrophils</th>
<th>Mast cells</th>
<th>T-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer16, pmol/mg protein</td>
<td>−0.81 (P &lt; .05)</td>
<td>−0.53</td>
<td>−0.40</td>
<td>0.46</td>
</tr>
<tr>
<td>Cer18, pmol/mg protein</td>
<td>−0.40</td>
<td>−0.14</td>
<td>0.63</td>
<td>0.58</td>
</tr>
<tr>
<td>Cer20, pmol/mg protein</td>
<td>−0.71</td>
<td>−0.59</td>
<td>−0.55</td>
<td>0.59</td>
</tr>
<tr>
<td>Cer22, pmol/mg protein</td>
<td>−0.72</td>
<td>−0.50</td>
<td>−0.58</td>
<td>0.34</td>
</tr>
<tr>
<td>Cer24, pmol/mg protein</td>
<td>−0.58</td>
<td>−0.10</td>
<td>−0.36</td>
<td>0.09</td>
</tr>
<tr>
<td>Cer24:1, pmol/mg protein</td>
<td>−0.61</td>
<td>−0.14</td>
<td>−0.33</td>
<td>0.22</td>
</tr>
<tr>
<td>Total ceramides, pmol/mg protein</td>
<td>−0.73</td>
<td>−0.36</td>
<td>−0.40</td>
<td>0.34</td>
</tr>
<tr>
<td>Sphingosine, pmol/mg protein</td>
<td>−0.66</td>
<td>−0.37</td>
<td>−0.24</td>
<td>0.02</td>
</tr>
<tr>
<td>S1P, pmol/mg protein</td>
<td>0.58</td>
<td>0.82 (P &lt; .05)</td>
<td>−0.13</td>
<td>−0.10</td>
</tr>
<tr>
<td>ASM, pmol/h/mg protein</td>
<td>−0.74</td>
<td>−0.60</td>
<td>−0.32</td>
<td>−0.42</td>
</tr>
</tbody>
</table>

ASM, Acid sphingomyelinase; S1P, sphingosine-1-phosphate.

DISCUSSION

In this study we characterized the PVAT that accumulates adjacent to human and murine AAA that has been shown to be correlated to the growth of AAA in diameter.15 We investigated human PVAT by elucidating the content of necrotic adipocytes, the type of infiltrating leukocytes, and the expression of proteases by immunohistochemistry and real-time quantitative PCR. Using lipidomics, we have also established a ceramide profile of PVAT in AAA. Adipocyte hypertrophy causes local hypoxia that in turn triggers infiltration of leukocytes. PVAT has been shown to be particularly susceptible to inflammation, which in turn induces vascular dysfunction.7,12,20 Our results show enhanced presence of inflammatory cells in PVAT of AAA, which is in agreement with what is observed in PVAT surrounding atherosclerotic aortae.1 Necrotic adipocytes that appear as perilipin-negative cells result in the recruitment of immune cells.18 We found abundant neutrophils, macrophages, mast cells, and T-cells in PVAT adjacent to AAA and in the near vicinity of necrotic adipose tissue. This has been shown that abnormal vessel wall of AAA contains abundant immune cells.5,27 However, high hemodynamic forces do not allow significant leukocyte rolling, adherence, or diapedesis at the aortic luminal surface. Likely, portals for their entry include capillary neovascularization that frequently develops within the adventitial layer of AAA as well as via post-capillary venules within nearby PVAT, followed by migration into the adjacent aneurysm. We also have made similar observations in thoracic aortic aneurysm (unpublished data) indicating that this phenomenon could play a general role in aneurysmal disease.

Leukocytes in PVAT may contribute to the production of proteases that can further damage the aortic wall. We found that cathepsin K and cathepsin S are elevated in PVAT adjacent to AAA compared with their levels in control aortae. Cathepsin S induces macrophage migration by degrading elastic fibers,28 and activity of cathepsin K shows to have a pivotal role in the elastase model of AAA.29 Our data, however, show that these proteases are highest expressed by aortic media in AAA, but their presence in PVAT may speculatively contribute to the pathogenesis of this disease.

The overwhelming, dominant causal agent in human AAA is tobacco smoking. PVAT from smokers has been shown to attract monocytes more avidly than adipocytes from nonsmokers.30 Cigarette smoke raises the level of ceramides that promotes sterile inflammation.31 Interestingly, ceramide and sphingomyelin, a ceramide metabolite and precursor, are both independent risk factors of atherosclerosis.32,33 Ceramide was shown to be critically involved in the signal transduction of proinflammatory mediators such as TNF-α and IL-1, in the synthesis of IL-6 and in the induction of programmed cell death, for instance after CD95 or DR5 stimulation.34 Interestingly, our data show that concentration of ceramides we measured in PVAT correlated positively with the T-cell marker CD3 in the adventitia of aortic wall of aneurysm but correlated negatively with neutrophils, mast cells, and macrophages. This suggests that ceramides are not involved in the recruitment of most of the inflammatory cells except for T-cells. Ceramides do not seem to be involved in the induction of necrosis either, a phenomenon that seems to be responsible for recruiting the macrophages and neutrophils into the PVAT. Instead, other factors should be considered and deserves attention in further studies. In fact, Guzik et al35 already showed that angiotensin II increases infiltration of T-cells into the PVAT.
Studies on cystic fibrosis tissues show that the increase of ceramide in the lung of mice results in an increased number of natural killer T-cells that mediate inflammation and tissue destruction. Thus, the primary role of ceramides in PVAT of AAA remains to be resolved. In addition, whether the natural killer T-cells are also increased in PVAT of AAA and play a role in the disease must be resolved.

Apoptosis of cells in the aneurysmal aorta is a hallmark of AAA, a finding in line with our present data. The expression of caspase 3 was significantly greater in AAA than in control aorta. In PVAT, however, only a slight upregulation of caspase 3 compared with that in the control aorta was observed.

CONCLUSIONS

We have shown that human and murine AAAs accumulate periaortic adipose tissue that is characterized by adipocyte necrosis, sterile inflammation, and robust expression of proteases. Our results suggest a role for this abnormal tissue in ongoing damage to the adjacent aneurysmal aortic wall.

AUTHOR CONTRIBUTIONS
Conception and design: MF, DW
Analysis and interpretation: MF, EV, EG, LJ, BK, MW, TL, DW
Data collection: MF, EV, EG, LJ
Writing the article: MF, EV, DW
Critical revision of the article: MF, EV, EG, LJ, BK, MW, TL, DW
Final approval of the article: MF, EV, EG, LJ, BK, MW, TL, DW
Statistical analysis: MF, EV, DW
Obtained funding: DW
Overall responsibility: DW

REFERENCES

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Additional material for this article may be found online at www.jvascsurg.org.
Supplementary Fig (online only). Gene expression of matrix metalloproteinase (MMP) 2 (A), MMP-8 (B), MMP-9 (C), MMP-12 (D), and tumor necrosis factor (TNF-α) (E) from human nonaneurysmal control aortas, abdominal aortic aneurysm (AAA) samples, and periaortic fat as determined by real-time polymerase chain reaction (PCR; n = 9 in all groups). *P < .05 and **P < .01. Open circle, ○ = Outlier greater than 1.5 times the interquartile range. Filled circle, • = Outlier greater than 3 times the interquartile range. A.adv, Aortic adventitia; A.med, aortic intima/media; PVAT, perivascular adipose tissue.

Supplementary Table (online only). Patient characteristics

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>70 ± 5.2</td>
</tr>
<tr>
<td>Male/female</td>
<td>16/3</td>
</tr>
<tr>
<td>AAA diameter, mm</td>
<td>63 ± 12.2</td>
</tr>
<tr>
<td>BMI</td>
<td>27 ± 3.1</td>
</tr>
<tr>
<td>Systolic and diastolic blood pressure, mm Hg</td>
<td>150 ± 18.3/90 ± 10.0</td>
</tr>
</tbody>
</table>

AAA, Abdominal aortic aneurysm; BMI, body mass index.