Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in postoperative insulin resistance

Short title: Adipokines in cardiac surgery patients

Jaromir Kremen, MD¹, Marketa Dolinkova, PhD¹, Jana Krajickova, Jan Blaha, MD², Katerina Anderlova, MD¹, Zdena Lacinova, PhD¹, Denisa Haluzikova¹,³, MD, Lenka Bosanska¹, MD, Martin Vokurka, MD⁴, Stepan Svacina¹, MD and Martin Haluzik, MD¹

¹,³rd Department of Medicine, ²Department of Anesthesia, Resuscitation and Intensive Medicine, ³Department of Sports Medicine, ⁴Department of Patophysiology

1st Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic

Corresponding author:

Martin Haluzik, MD, PhD

3rd Department of Medicine, 1st Faculty of Medicine, Charles University

U Nemocnice 1

128 08, Prague 2

Czech Republic

Phone: +420-224962908

FAX: +420-224919780

E-mail: mhalu@lf1.cuni.cz

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ABSTRACT

Context: Hyperglycemia and insulin resistance frequently occur in critically ill patients even without history of diabetes.

Objective: To study the role of adipose tissue hormonal production in the development of insulin resistance in cardiac surgery patients.

Participants/interventions/settings: 15 patients with elective cardiac surgery underwent blood sampling before, at the end and 6, 12, 24, 48, 120 hours after the end of operation; epicardial and subcutaneous adipose tissue sampling at the beginning and at the end of operation in the Department of Cardiac Surgery.

Main outcome measures: Serum concentrations, subcutaneous and epicardial adipose tissue mRNA expression of interleukin-6, MCP-1, TNF-α, leptin, resistin, adiponectin. Subcutaneous and epicardial adipose tissue mRNA expression of CD14, CD45, CD68.

Results: The rate of insulin infusion required to maintain euglycemia increased up to 7-fold 12 hours after the operation suggesting the development of insulin resistance. Serum IL-6 levels increased 43-fold 12 hours after operation. MCP-1 peaked 6-fold at the end of operation. Smaller peaks of TNF-α and leptin appeared 6 and 12 hours after operation, respectively. Resistin levels peaked 4-fold 24 hours after operation, adiponectin levels were not significantly affected. TNF-α and CD45 mRNA expression increased markedly during the operation in subcutaneous adipose tissue. IL-6, resistin and MCP-1 mRNA expression increased in both subcutaneous and epicardial adipose tissue. Leptin, adiponectin, CD14 and CD68 mRNA expression did not change significantly.

Conclusions: Both subcutaneous and epicardial adipose tissue is a source of proinflammatory cytokines in cardiac surgery patients and may contribute to the development of postoperative insulin resistance.
Introduction

Increased incidence of obesity and type 2 diabetes worldwide stimulated intensive research focusing on the detailed etiopatogenesis of its relationship. During the last decade a lot of new knowledge have been gained in this field including the discovery of endocrine function of adipose tissue (1; 2). It is now generally accepted that adipose tissue secretes numerous hormones and cytokines that can have both insulin resistance-inducing and insulin-sensitizing effects (3; 4). Endocrine dysfunction of adipose tissue together with an excessive ectopic lipid storage in non-adipose tissues such as liver and muscle is now considered the major player in the etiopatogenesis of obesity-related insulin resistance (5; 6).

Increased blood glucose levels and decreased sensitivity to insulin effects frequently occur also in critically ill patients even without previous history of diabetes mellitus (7). Numerous studies have documented that increased blood glucose levels worsen morbidity and mortality in critically ill patients (8; 9) and that intensive insulin therapy aimed at maintaining euglycemia markedly improves the outcome of these patients (10; 11).

The etiopatogenesis of insulin resistance in critically ill patients is still only partially understood and likely includes both some of the mechanisms analogical or similar to that of obesity-induced insulin resistance and other processes. Major patophysiological conditions underlying hyperglycemia in critical illness include enhanced hepatic gluconeogenesis, impaired insulin secretion and decreased insulin sensitivity due to anti-insulin effects of stress hormones and proinflammatory cytokines (7; 9; 12). The exact mechanisms at the molecular level still remain to be elucidated.

While the involvement of adipose tissue hormones in the obesity-induced insulin resistance has been studied extensively (3; 4) there is scarce information about its changes in critically ill patients. Recently, epicardial adipose tissue has been identified as a source of several proinflammatory cytokines and have been implicated as a possible player in the development of coronary artery disease (13; 14). Here we studied the dynamic changes of several proinflammatory and anti-
inflammatory adipose tissue-derived hormones both on systemic and local level as measured by changes of its mRNA expression in subcutaneous and epicardial adipose tissue. We demonstrate that both epicardial and subcutaneous adipose tissue becomes a significant source of proinflammatory factors after major elective cardiac surgery operation and thus may contribute to the development of insulin resistance in these patients.

MATERIAL AND METHODS

Study subjects
15 patients (5 men and 10 women; mean age 68±3 years; mean BMI 26.6±1.2 kg/m²) who had major elective cardiac surgery operation (10 patients with aorto-coronary bypass, 5 with valvular plastique) were included into the study. 3 of patients had type 2 diabetes on the insulin therapy, 8 of the patients had arterial hypertension. None of the patients had malignant tumor, thyroid disease or acute infectious disease. All patients on the ICU were treated by continuous intravenous insulin infusion (Actrapid HM, Novo Nordisk, Baegsvard, Denmark) using internal glucose control protocol to maintain normoglycemia (4.4-6.1 mmol/l). Written informed consent was signed by all participants before being enrolled in the study. The study was approved by the Human Ethical Review Committee, 1st Faculty of Medicine and General University Hospital, Prague, Czech Republic and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

Anthropometric examination and sampling

Anthropometric examination of the patients was performed at basal state one day before operation. All subjects were measured and weighted and BMI was calculated. Blood samples for hormonal measurement were taken at basal state (before the start of anesthesia), at the end of operation and 6, 12, 24, 48 and 120 hours after the end of operation,
respectively. Serum was obtained by centrifugation and the samples were subsequently stored in aliquots at -70 °C until further analysis.

Samples of the subcutaneous and epicardial adipose tissue for mRNA expression analysis were taken at the beginning and before the end of surgery. Subcutaneous samples were from the thoracic region. All of the samples (both at the beginning and at the end of operation) were taken from approximately same location in all of the patients. The samples were obtained from the tissue that has not been previously traumatized mechanically or by cauterization to avoid the influence of local damage on tissue parameters. Tissue samples were collected to RNAlater reagent (Qiagen, Germany) and stored at -70 °C until further analysis. The average time between the withdrawal of the sample at the beginning and at the end of operation was 252±27 minutes.

Blood glucose was monitored in hourly intervals during first 48 hours of stay on ICU and in 1-4 hours intervals based on the actual glucose levels afterwards. Insulin infusion was started at the time of admission to ICU (within 5 minutes after the end of operation). Insulin infusion rate was adjusted according to internal glucose control protocol aiming to maintain blood glucose within euglycemic limits (4.4-6.1 mmol/l).

**Hormonal and biochemical assays**

Blood glucose was measured on ABL 700 analyzer (Radiometer Medical A/S, Copenhagen, Denmark). Serum concentrations of insulin, interleukin-6, TNF-α, leptin and MCP-1 were measured using Human serum adipokine LINCOplex Kit (panel B) on Luminex®200 instrument (Linco Research, USA). Sensitivity was 1.6 pg/ml for IL-6, 85.4 pg/ml for Leptin, 0.14 pg/ml for TNF-alpha, 0.14 pg/ml for MCP-1 and 50.9 pg/ml for Insulin. Intra- and interassay variability of kit were 1.4-7.9% and <21%, respectively.
Serum adiponectin concentrations were measured by commercial RIA kit (Linco Research, St. Charles, Missouri, USA). Sensitivity was 1.0 ng/ml, and the intra- and interassay variability were 1.8% and 9.3%, respectively. Serum resistin concentrations were measured by commercial ELISA kit (BioVendor, Czech Republic). Sensitivity was 0.2 ng/ml, and the intra- and interassay variability were 3.1% and 6.5%, respectively. Serum cortisol concentrations were measured using Cortisol RIA kit (Immunotech, Czech Republic). Sensitivity was 10 nmol/l, and the intra- and interassay variability was 5.8% and 9.2%, respectively.

**Determination of mRNA expression:**

Approximately 100mg of tissue was collected to 1ml of RNA stabilization Reagent (RNAlater, Qiagen, Germany) and stored at -80°C until further analysis. Total RNA was extracted from subcutaneous and epicardial adipose tissue by homogenization with an ULTRA-TURRAX® T 18 basic (IKA® Werke GmbH, Staufen, Germany) using RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). The RNA concentration was determined from absorbance at 260 nm (BioPhotometer, Eppendorf AG, Germany). All samples had a 260/280 nm absorbance ratio 1,89 ± 0,1. The integrity of the RNA was checked by visualization of 18S and 28S ribosomal bands on 1% agarose gel with an ethidium bromid. 0,1-1µg of total RNA was used for reverse transcription to synthesize the first strand cDNA using the oligo(dT)₁₈ primers following the instructions of the RevertAid First Strand cDNA synthesis kit (Fermentas Life Science, Lithuania). Measurements of adiponectin and leptin gene expression were performed on a LightCycler 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany), using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, GmbH, Mannheim, Germany) and specific DNA primers. Measurements of resistin, interleukin-6, MCP-1, TNF-α, CD14, CD45 and CD68 gene expression were
performed on an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix, NO AmpErase® UNG and specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA).

All PCRs for each gene were amplified separately. Controls with no template cDNA were performed with each assay and all samples were run at least in duplicates. The increase in fluorescence was measured in real time and data were obtained as threshold cycle (C_T) values. To compensate for variations in input RNA amounts and efficiency of reverse transcription, beta-2-microglobulin was used as an endogenous reference and results were normalized to these values. Relative gene expression of genes was calculated using the formula $2^{-\Delta\Delta C_T}$ (cytokine-CT B2M).

**Statistical analysis**

The statistical analysis was performed on SigmaStat software (Jandel Scientific, USA). The results are expressed as means ± standard error means (SEM). Changes of hormonal levels and gene expression during perioperative and postoperative state, respectively, were evaluated using RM ANOVA or paired t-test as appropriate.

**RESULTS**

**Blood glucose levels and insulin requirements**

Mean blood glucose level during the first 48 hours of stay on ICU was 6.5±0.13 mmol/l, mean 48-hours insulin consumption was 204±37.6 IU (Table 1). Average insulin infusion rate was 4.01± 0.77 IU/h (Table 1). Glucose concentrations, insulin infusion rate and serum insulin levels during first 48 hours of ICU stay are shown in Figure 1.

**Serum hormonal and cytokine concentrations**
Serum concentrations of insulin, resistin, interleukin-6, MCP-1, TNF-α, adiponectin, leptin and cortisol at basal state and during postoperative period up to 120 hours after operation are shown in Figure 1 and Figure 2, respectively. All of the serum hormonal concentrations with the exception of adiponectin were significantly affected by the operation. The time pattern of changes of insulin and interleukin-6 was very similar. Both insulin and interleukin-6 levels increased moderately after the operation, peaked 6 and 12 hours after operation, respectively and remained 2-3 times elevated even 120 hours after operation (Figure 1 and 2). Serum TNF-α showed a two-peak pattern: with increments at 6 and 20 hours after the end of operation (Figure 2). Leptin levels doubled 6 hours after the end of operation, peaked 12 and 24 hour after operation, respectively and normalized until 120 hour after operation (Figure 2). MCP-1 levels peaked at the end of operation (3-fold increase over the baseline) and returned to basal levels 48 hours after the operation (Figure 2). Resistin levels showed the slowest pattern of increase with peaks 24 and 48 hours after operation, respectively, remaining still 2-fold elevated 120 hours after the end of operation (Figure 2). In contrast, serum adiponectin concentrations only tended to decrease during the operation and returned to preoperative levels 120 hours after the end of operation (Figure 2). None of the changes of adiponectin levels reached the statistical significance. Serum cortisol levels increased after the end of operation, peaked 12 hours after the end of operation and normalized 120 hours after the end of operation (Figure 2).

Changes of mRNA expression of selected adipose tissue-derived hormones and cytokines
At baseline, TNF-α mRNA expression was significantly higher in epicardial relative to subcutaneous adipose tissue, while no significant differences between the two adipose tissue depots were found for leptin, adiponectin, resistin, MCP-1 and interleukin-6 expression (Figure 3). In contrast, operation induced major increases in interleukin-6 and MCP-1 mRNA
in both subcutaneous and epicardial adipose tissue (Figure 3). Resistin mRNA expression also significantly increased postoperatively in both subcutaneous and epicardial adipose tissue, however this increase was quantitatively less significant relative to interleukin-6 and MCP-1. TNF-α mRNA expression increased in subcutaneous, but did not significantly change in epicardial adipose tissue (Figure 3). No significant changes in subcutaneous or epicardial adipose tissue expression of leptin or adiponectin mRNA were detected (Figure 3).

Changes of mRNA expression of immunocompetent cells markers

mRNA expression of CD14 (macrophage and monocyte marker), CD45 (monocyte, T-lymphocyte, B-lymphocyte and granulocyte marker) and CD68 (macrophage, monocyte and polymorphonuclear cells marker) was measured in both subcutaneous and epicardial adipose tissue at the beginning and at the end of operation. All three markers were detectable in both subcutaneous and epicardial adipose tissue indicating the presence of immunocompetent cells in both adipose tissue depots (Figure 4). The presence of CD68-positive cells was further confirmed by immunohistochemistry with anti-CD68/KP1 antibody (data not shown). At baseline, CD45 and CD68 mRNA expression was higher in epicardial vs. subcutaneous depot (Figure 4). Operation increased CD45 expression in subcutaneous adipose tissue relative to baseline values, but did not affect its mRNA expression in epicardial adipose tissue or CD14 and CD68 mRNA in any adipose tissue depot (Figure 4).

DISCUSSION

The most important finding of this study is that both epicardial and subcutaneous adipose tissue can produce significant amounts of proinflammatory factors after activation of immune system and stress axis by major cardiac surgery operation. Epicardial adipose tissue as a source of inflammatory mediators under basal conditions has been identified previously (13;
14) however it has not yet been described how adipose tissue responds to major stressor such as cardiac surgery operation.

Here we measured classical proinflammatory cytokines such as TNF-α, more recently discovered substances such as resistin and MCP-1 and the only known adipose tissue-derived factor with major insulin-sensitizing and anti-inflammatory properties – adiponectin together with circulating insulin and cortisol levels. Operation markedly increased the amount of exogenous insulin necessary to maintain euglycemia as well as circulating insulin levels suggesting the development of insulin resistance. **It has to be noted that the level of insulin resistance has not been directly measured in this study, but its presence in the postoperative period in surgical patients have been documented by others previously** (15; 16). Circulating concentrations of all pro-inflammatory factors increased markedly during the postoperative period. Even more interestingly, the increase of the above mentioned hormones not only appeared on the systemic level but also on the level of mRNA expression in adipose tissue as early as after 4 hours of operation.

Another important finding of this study is the time pattern of the changes of proinflammatory cytokines levels. MCP-1 concentrations peaked as early as after the end of the operation followed by later peaks of TNF-α, interleukin-6 and resistin, respectively. These findings are in agreement with the concept of infiltration of adipose by macrophages that is initiated by the adipose tissue production of MCP-1 (17; 18). Activated macrophages that migrate to the adipose tissue produce proinflammatory cytokines and thus markedly contribute to the overall immune system activation. Excessive infiltration of adipose tissue by activated macrophages in obesity is considered one of the reasons for increased production of proinflammatory adipokines seen in obesity and type 2 diabetes (17-19). Here we measured mRNA expression of three immunocompetent cells markers CD14, CD45 and CD68 to assess the role of this process in the production of proinflammatory cytokines. We found that mRNA expression of
CD45 – the marker of the presence of monocytes, T- and B-lymphocytes and granulocytes increased in subcutaneous but not epicardial adipose tissue at the end of operation. In contrast, other two markers CD14 and CD68 were not affected by the operation. This suggests that even in relatively lean subjects participating in our study significant amount of immunocompetent cells is present in both subcutaneous and epicardial adipose tissue at the beginning of operation. The lack of acute increase of two of three immunocompetent cells markers at the end of operation may indicate that both immunocompetent cells chronically residing in adipose tissue and those migrating there as a result of operation are involved in the increased production of proinflammatory cytokines by adipose tissue. Taken together, our data suggest that adipose tissue may represent an important source of immunocompetent cells used to respond to different forms of stressors including metabolic stress in obesity and operation stress in cardiac surgery patients as demonstrated here.

While the possible involvement of epicardial adipose tissue in the production of interleukin-6, TNF-α and MCP-1 has been described previously (13; 20), its role in the production of resistin has not been extensively studied by far. In the only report available, Baker et al. found resistin expression in epicardial adipose tissue comparable to that in abdominal subcutaneous and visceral adipose tissue and higher than in gluteal subcutaneous adipose tissue (14). Here we did not see significant differences in resistin mRNA expression between epicardial and subcutaneous adipose tissue from the thoracic region and found a postoperational increase in its expression in both subcutaneous and epicardial adipose tissue.

Resistin was originally discovered as an adipocyte-derived hormone increased in obesity and was suggested to link obesity to insulin resistance (21). Further studies revealed that its major role in humans may lie rather in its proinflammatory than insulin resistance-inducing action and that in humans it is produced rather by activated immunocompetent cells than adipocytes (22-25). Here we show for the first time that resistin behaves similarly to other
in proinflammatory cytokines being increased by operational stress. Thus at least in cardiac surgery patients it may in concert with other proinflammatory factors contribute to the development of insulin resistance. The exact source of resistin and other proinflammatory cytokines within the adipose tissue (adipocytes vs. immunocompetent cells in stroma-vascular fraction) has not been addressed in this study. However, our preliminary data on another group of cardiac surgery patients indicate that resistin is produced almost exclusively by stroma-vascular fraction (Dolinkova et al, unpublished data) which is in agreement with previously published data in visceral adipose tissue of lean humans (25). Taken together, the finding of increased adipose tissue mRNA expression of proinflammatory cytokines underlines its possible contribution in the development of insulin resistance of critically ill patients.

In addition to proinflammatory mediators, adipose tissue also produces adiponectin - a protein hormone with significant insulin-sensitizing, anti-inflammatory and anti-atherosclerotic properties (4; 26; 27). In contrast to proinflammatory factors such as resistin, interleukin-6, TNF-α and MCP-1 markedly affected by the cardiac surgery operation no significant changes were detected in serum adiponectin levels or its adipose tissue mRNA expression. This suggests that in contrast to resistin, interleukin-6, TNF-α and MCP-1 the changes in circulating adiponectin levels are probably not involved in the etiopatogenesis of insulin resistance in critically ill patients. However, it has to be noted that by measuring total adiponectin levels we may have missed the changes of its circulating fractions that can also modulate insulin sensitivity as was demonstrated previously (28).

Despite an attractive hypothesis of significant role of adipose tissue-derived factors as important players in the insulin resistance of critically ill patients it is important to interpret our findings cautiously. Firstly, mRNA expression of adipose tissue-derived factors was measured only in subcutaneous adipose tissue in thoracic region and epicardial adipose tissue
in our study and it is unknown if such changes also appear in other fat deposits. Secondly, circulating monocytes and macrophages activated by the operation can also significantly contribute to the circulating pool of proinflammatory cytokines. It is also important to note that many other factors in addition to proinflammatory cytokines such as cortisol, catecholamines, growth hormone and other stress-related factors can significantly contribute to the development of insulin resistance in critically ill patients (29). For example: cortisol has been found to induce insulin resistance in both muscle (30) and liver (31) and its decrease by adrenalectomy under experimental conditions markedly decreased hyperglycemia and improved insulin sensitivity in different rodent models of insulin resistance (32; 33). Conversely, increased cortisol levels in patients with endogenous hypercortisolism such as Cushing syndrome induce insulin resistance which disappears after normalization of cortisol levels by appropriate treatment (33; 34). Cortisol levels were significantly elevated in the postoperative period in our study and therefore very likely contributed to the development of insulin resistance together with other stress-induced hormones (29).

In summary, we have demonstrated that both subcutaneous and epicardial adipose tissue becomes an important source of proinflammatory factors in patients with major cardiac surgery operation. These factors together with other hormonal and metabolic changes contribute to the development of insulin resistance in these patients. Our finding suggests that therapeutic approaches suppressing proinflammatory factors production in adipose tissue may represent a new modality of prevention and/or treatment of insulin resistance in critically ill patients.

ACKNOWLEDGEMENTS

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REFERENCES

Figure Legends

Figure 1
Blood glucose levels, insulin infusion rate and serum insulin levels in cardiac surgery patients.
Samples for insulin measurements were taken at baseline (before the start of anesthesia, marked as -6 hours in graph), immediately after the end of operation (time 0 in graph) and 6, 12, 24, 48 and 120 hours after the end of operation, respectively. Samples for blood glucose measurements were taken at baseline (before the start of anesthesia, marked as -6 hours in graph) than hourly for first 48 hours after the end of operation and 1-4 hourly for next 72 hours. For the clarity sake the graph shows only 4-hourly values for the first 48 hours period and 12-hourly values for the next 72 hours period. Values are mean ± S.E. with n=15/group. Statistical significance is from RM ANOVA: * indicates p < 0.05 vs. baseline value.

Figure 2
Serum concentrations of resistin, interleukin-6, MCP-1, TNF-α, adiponectin, leptin and cortisol in cardiac surgery patients.
Samples were taken at baseline (before the start of anesthesia, marked as -6 hours in graph), immediately after the end of operation (time 0 in graph) and 6, 12, 24, 48 and 120 hours after the end of operation, respectively. Values are mean ± S.E. with n=15/group. Statistical significance is from RM ANOVA: * indicates p < 0.05 vs. baseline value.

Figure 3
mRNA expression of resistin, interleukin-6, MCP-1, TNF-α, adiponectin and leptin in subcutaneous and epicardial adipose tissue samples taken and the beginning (filled bars) and at the end of operation (open bars).

Values are mean ± S.E. with n=15/group. Statistical significance is from paired or un-paired T-test: * indicates p < 0.05 vs. baseline expression in the same adipose tissue depot,
+indicates p < 0.05 for subcutaneous vs. epicardial adipose tissue taken at the beginning of operation

Figure 4
mRNA expression of CD14, CD45 and CD68 in subcutaneous and epicardial adipose tissue samples taken and the beginning (filled bars) and at the end of operation (open bars).

Values are mean ± S.E. with n=15/group. Statistical significance is from paired or un-paired T-test: * indicates p < 0.05 vs. baseline expression in the same adipose tissue depot,
+indicates p < 0.05 for epicardial vs. subcutaneous adipose tissue taken at the beginning of operation
Table 1:

Clinical and biochemical characteristics of the cardiac surgery patients. Expressed as n (number of subjects) or mean ± S.D.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Number of subjects (male/female)</td>
<td>15 (5/10)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.2±2,54</td>
</tr>
<tr>
<td>Age (years)</td>
<td>68±10</td>
</tr>
<tr>
<td>Baseline blood glucose (mmol/l)</td>
<td>6.61±2,31</td>
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<tr>
<td>Mean blood glucose (mmol/l)</td>
<td>6.58±0,40</td>
</tr>
<tr>
<td>Insulin dose (IU/24 hrs.)</td>
<td>102,5±58,2</td>
</tr>
<tr>
<td>Insulin infusion rate (IU/h)</td>
<td>4.02± 2,35</td>
</tr>
</tbody>
</table>
Blood glucose levels

Insulin infusion rate

Serum insulin levels

Kremen et al. Figure I
Kremen et al.  Figure 3

**Resistin**

- Subcutaneous adipose tissue
- Epicardial adipose tissue

**IL-6**

- Subcutaneous adipose tissue
- Epicardial adipose tissue

**MCP-1**

- Subcutaneous adipose tissue
- Epicardial adipose tissue

**TNF-α**

- Subcutaneous adipose tissue
- Epicardial adipose tissue

**Adiponectin**

- Subcutaneous adipose tissue
- Epicardial adipose tissue

**Leptin**

- Subcutaneous adipose tissue
- Epicardial adipose tissue

*Start of operation vs. End of operation*
Kremen et al. Figure 4