The role of gut microbiota in human metabolism
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Citation for published version (APA):
Vrieze, A. (2013). The role of gut microbiota in human metabolism

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CHAPTER 7

VISCERAL ADIPOSE TISSUE BACTERIAL CONTENT IS ASSOCIATED WITH ALTERED INTESTINAL MICROBIOTA COMPOSITION AND MACROPHAGE RECRUITMENT IN OBESE HUMANS

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Manuscript in preparation
Abstract

Background
Inflammation of visceral fat is a key step in the development of insulin resistance and subsequent type 2 diabetes, yet the pathophysiological trigger remains poorly understood. As the intestinal microbiota is known to play a pivotal role in metabolism, the aim of our study was to investigate whether a) humans with chronic visceral adipose tissue inflammation are characterized by increased endotoxemia and altered intestinal microbiota composition, and b) whether intestinal microbial DNA can be detected in visceral adipose tissue specimens.

Methods
Treatment-naïve healthy obese subjects scheduled for elective laparoscopic cholecystectomy were included. Based on plasma LBP, we identified groups with high and low endotoxemia. At the day of surgery, patients collected stool samples for global and deep intestinal microbiota composition as determined by phylogenetic microarray analysis. During surgery, visceral adipose tissue was collected in sterile endotoxin-free containers and immediately processed for macrophage (CD68) crown-cell staining and bacterial DNA content analysis. Denaturing gradient gel electrophoresis (DGGE) followed by DNA sequencing was used to characterize the microbial DNA in the visceral adipose tissue.

Results
Endotoxemia was associated with visceral adipose tissue macrophage accumulation (low LBP group: 4.1±1.5 vs. high LBP group: 8.5±2.1 macrophages/cm², p<0.0001). Plasma LBP correlated with the amount of crown-like structures (Spearman’s rho=0.4, p<0.05). A trend towards increased Bacteroidetes was found in stool samples of subjects with high LBP. There was no difference in the amount of total bacterial DNA in visceral adipose tissue. Ralstonia species were predominantly present in visceral adipose tissue and were significantly increased in subjects with low LBP levels compared to the high LBP group (low LBP group: 84.6±7.1 vs. high LBP group: 59.5±8.6 Ralstonia /total bacterial DNA, p<0.05).

Conclusions
Endotoxemia is associated with the level of visceral adipose tissue macrophage accumulation whereas Ralstonia DNA in visceral adipose tissue was inversely related to macrophage count. In conclusion, our data imply that intestinal microbiota may play a role in chronic inflammation of human visceral adipose tissue.
Introduction

We are in the midst of a worldwide epidemic of obesity, which is a known major risk factor for the development of common medical conditions such as type 2 diabetes, dyslipidemia and subsequent cardiovascular disease (1). During the past decade, it has been acknowledged that metabolically healthy and unhealthy obese phenotypes exists based on the presence of chronic inflammation (2). It is widely acknowledged that obesity and subsequent insulin resistance are closely related to the presence of adipose tissue inflammation (3;4). Since adipose tissue is an important organ producing various inflammation cytokines (3), there is ample evidence that visceral adipose tissue and to a lesser extent subcutaneous adipose tissue drives deterioration of insulin resistance (4;5). More recently, the amount of visceral adipose tissue macrophages and crown-like structures (CLS, or accumulated CD68 positive macrophages) was found to correlate with inflammatory gene expression (6), suggesting a role for the innate immune system in this process (7;8). It was originally thought that this macrophage infiltration was catalyzed by necrosis of foam cells (9), but the exact pathophysiological mechanisms driving this immunologic inflammatory process remain to be elucidated.

Endotoxemia is characterized by gram-negative bacterial capsule fragments in the plasma, and is linearly associated with the concentration of lipopolysaccharide binding protein (LBP) in plasma. As such, plasma LBP is regarded as a marker of chronic inflammation associated with the development of obesity and insulin resistance in both animals and humans (10-13). There is increasing evidence that intestinal microbiota might contribute to host metabolism and obesity (14;15), a process which is thought to stem from impaired gut barrier function in obese subjects (16). The subsequent increased plasma endotoxin concentration in the portal vein may exceed the clearance capacity of the hepatic Kupffer cells, resulting in systemic endotoxemia and low grade chronic inflammation (17). Indeed, recent data indicate that exogenous stimuli, such as dietary interventions, directly drive gut microbial composition and subsequent intestinal permeability (18;19). For example, postprandial chylomicronemia is associated with an increased plasma endotoxin
load (20) and recent data suggest that a local inflammatory response can be elicited by LPS-producing gram-negative bacteria and subsequent macrophage influx in the visceral adipose tissue of mice (21). Recently, Amar et al. showed in an animal model of insulin resistance that adipose tissue macrophages contain bacterial DNA that originated from the intestine, which was directly linked to low grade chronic inflammation (21). A key question however remains which intestinal bacterial species drive endotoxemia and subsequent visceral adipose tissue inflammation in obese humans. Thus, the aim of our study was to investigate whether a) humans with and without inflamed visceral adipose tissue have different plasma levels of bacterial products and altered intestinal microbiota composition and b) whether intestinal microbial DNA can be detected in visceral adipose tissue.

Methods

Participants
This was a multicenter trial in which several hospitals within the Amsterdam area were involved. Caucasian subjects scheduled for elective laparoscopic cholecystectomy were screened and asked for consent by the attending surgeon. Inclusion criteria were age between 18-75 years and body-mass index (BMI) between 18-45 kg/m². Exclusion criteria were malignancy, diabetes, generalized inflammation and use of probiotics and/or antibiotics in the past three months (22). Written informed consent was obtained from all subjects. The study was conducted at the Flevo hospital (Almere), Sint Lucas Andreas hospital (Amsterdam) and Academic Medical Center (Amsterdam), in accordance with the Declaration of Helsinki (updated version 2008). The study was approved by Institutional Review Boards of each involved hospital and registered with the Dutch Trial Register (NTR 2335).

Study protocol
Prior to surgery, anthropometric measurements and (overnight fasted) blood sampling was performed and participants collected a stool sample. During the surgical procedure visceral and subcutaneous fat biopsies were simultaneously obtained and
directly collected in sterile, RNase-, DNase- and pyrogen free microtubes (Eppendorf) either stored in formalin 4% or promptly frozen in liquid nitrogen (LN₂) to be stored at -80°C for later analyses. Participants could continue their own diet, but were asked to fill out a weekly online nutritional diary (www.dieetinzicht.nl) to monitor caloric intake including the amount of dietary carbohydrates, fats, proteins and fibres.

**Biochemistry**
Fasting plasma samples were obtained for measurement of metabolic parameters (glucose, insulin levels, lipid profile and liver enzymes) and inflammation parameters C-Reactive Protein (CRP, Roche Diagnostics) and lipopolysaccharide binding protein (LBP, Hycult ELISA). Total cholesterol, low density lipoprotein cholesterol (LDLc), high density lipoprotein cholesterol (HDLc) and triglycerides (TG) were measured by using commercially available enzymatic assays (Randox, USA and Daiichi, Japan). All analyses were performed using a Cobas Mira autoanalyzer (Horiba, France). Insulin resistance was determined using the homeostasis model assessment (HOMA).

**Intestinal microbiota analyses**
Study subjects collected a morning stool sample to determine the intestinal microbiota composition. Samples were collected into a plastic container, immediately frozen at -20°C and transferred to -80°C within a week. The microbiota composition of the fecal samples was determined by analyzing 16S rRNA-based signatures using the Human Intestinal Tract Chip (HITChip), a custom-made Agilent microarray (Agilent Technologies, Palo Alto, CA, USA) containing approximately 5,500 specific oligonucleotide probes that cover over 1,000 intestinal phylotypes (23;24).

**Histological localization of macrophages in visceral adipose tissue sections (CD 68 staining)**
CD68 staining in adipose tissue was performed to detect macrophage infiltration. Adipose tissue embedded in paraffin was de-paraffinized and hydrated using xylene – alcohol series. The endogenous peroxidase activity was blocked by incubating with hydrogen peroxide and heat treated for antigen retrieval. After blocking with Ultra V block (Thermo), it was incubated for 1 hour with mouse anti-human CD-68 (clone
PG-M1, Dako) against macrophages at a dilution of 1:200. Slides were developed with Bright vision anti- mouse poly-horse radish peroxidase (HRP) conjugated antibody (Immunologic) and diaminobenzidine (DAB+, Immunologic). Nuclei were counterstained with 1:10 diluted hematoxilin (Mayer’s composition), dehydrated and mounted using Pertex Mounting Media (Leica). Images of CD68 stained adipose tissue samples (n= 5 per patient with three sections per slide) were obtained using a bright field microscope (Leica systems) at a 10x magnification for counting crown like structures (CLS) and 40x magnification for individual macrophages using Leica application suite 3.8 software.

**Bacterial DNA in visceral adipose tissue**

Genomic DNA of both prokaryotic and eukaryotic origin was isolated from visceral adipose tissue according to Zoetendal et al. (25). Briefly, tissues were first treated with a mix of SDS and proteinase K at 55°C and homogenized by mechanical disruption in the presence of phenol and zirconia glass beads (1mm) in the FAST Prep-24 (MP Biomedical). The released genomic DNA was further extracted with a number of phenol/chloroform extractions and precipitated in the presence of absolute ethanol. The prokaryotic fraction was studied by using a range of 16S rRNA gene specific primers and assays. From the minute amounts of bacterial genomic DNA, full-length16S rDNA amplicons were generated in a PCR by using primers Bact-27F (5’GTTTGATCCTGGCTCAG-3’) and Prok-1392R (5’GCCCGGGAACGTATTCACCG-3’) using PCR conditions described by Rajilic-Stojanovic et al (24). The resulting amplicons were used as imput for a nested PCR using primers 968-GC-F (5’CGCCCGGGGCGCCCGGCGGGGGGGCAGGGGGAAACGCGGCGAAACCTTAC) and 1392R (26), generating fragments fit for a diversity analysis by DGGE (denaturing gene gel electrophoresis) using conditions described by Heilig et al. (27). The dominant band appearing in the DGGE analyses was identified by cloning the DGGE amplicon in a pGEM-T easy vector (Promega, Leiden, The Netherlands) and transforming them to Stratagene E. coli XL-1 Blue competents cells (Agilent Technologies, Amstelveen, The Netherlands) according to the manufacturers’ specifications. Clones containing the right size insert and migrating to the same position as the dominant band in the DGGE gel were subjected to Sanger sequence analysis (GATC Biotech, Konstanz, Germany). Sequences were
identified by performing a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis of the dominant band appearing in DGGE showed highest similarity to sequences of *Ralstonia* species. To estimate the *Ralstonia*-like bacteria within the total bacterial fraction, two nested quantitative PCR assays were performed on the previously generated 16S rDNA amplicons. *Ralstonia* being a member of the group of *Burkholderiales*, was detected in a *Burkholderia* specific PCR (28). Total bacteria qPCR was performed as described by Zweilehner et al. (29) and the fraction *Burkholderia* to total bacteria within the 16S rDNA amplicons could be calculated.

**Statistical analysis**

Statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, USA). Data are expressed as median with range. For comparison of two groups Mann Whitney test or Wilcoxon Signed rank test was used based on distribution. Statistical difference was considered significant when p<0.05. The analysis of the HITChip microarrraays was carried out essentially as described previously (23) with nlme package in R (30). Differences between high and low LBP were computed using linear mixed model, and the p-values were then corrected for multiple comparisons by q-value package (31;32).

**Results**

**Clinical data**

We collected clinical data as well as plasma, fecal and subcutaneous as well as visceral adipose tissue samples in a random set of 47 patients. From this group, we selected 12 patients with the highest LBP plasma levels and 12 patients with the lowest LBP plasma levels. Table 1 shows the baseline characteristics for both groups. As indicated, there were no between-group differences in age, glucose-, insulin levels, HOMA and lipid profiles and/or daily dietary intake. However, the patients in the high LBP group had a higher BMI (p=0.02), higher leukocyte count (p=0.02) and a higher CRP (p=0.06) compared to the low LBP group. Moreover, no differences in diet composition/ daily caloric intake were observed between the two groups.
Table 1. Baseline Characteristics of study subjects

<table>
<thead>
<tr>
<th></th>
<th>Low LBP (N=12)</th>
<th>High LBP (N=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBP (µg/ml)</td>
<td>15.1 (2.2-20.6)</td>
<td>57.4 (50.3-159.7) *</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 (25-71)</td>
<td>46 (32-58)</td>
</tr>
<tr>
<td>Body-mass index (kg/m²)</td>
<td>26.0 (19.5-36.8)</td>
<td>32.9 (24.4-45.2) *</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/liter)</td>
<td>4.9 (4.0-5.7)</td>
<td>5.2 (4.3-6.0)</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>37.0 (4-87)</td>
<td>42.5 (7-117)</td>
</tr>
<tr>
<td>Homeostatic Model Assessment</td>
<td>1.2 (0.1-3.2)</td>
<td>1.5 (0.3-4.3)</td>
</tr>
<tr>
<td>Cholesterol (mmol/liter)</td>
<td>5.0 (3.4-5.6)</td>
<td>5.0 (3.6-6.4)</td>
</tr>
<tr>
<td>HDLc</td>
<td>1.4 (0.9-3.0)</td>
<td>1.3 (0.9-1.7)</td>
</tr>
<tr>
<td>LDLc</td>
<td>2.2 (1.8-3.9)</td>
<td>3.5 (2.1-4.3)</td>
</tr>
<tr>
<td>TG</td>
<td>1.0 (0.4-2.2)</td>
<td>1.2 (0.5-2.0)</td>
</tr>
<tr>
<td>Leucocytes (x10⁹/L)</td>
<td>5.5 (2.3-9.4)</td>
<td>7.6 (4.9-21.6) *</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.1 (0.6-21.7)</td>
<td>6.3 (1.3-140.0)</td>
</tr>
<tr>
<td>Caloric intake (kcal/day)</td>
<td>2065 (1490-3050)</td>
<td>2180 (1545-3600)</td>
</tr>
</tbody>
</table>

Data are presented as median (range), * p<0.05

Inflammation of visceral adipose tissue

Crown-like structures, the number of macrophages and the amount of adipocytes were quantified in visceral adipose tissue sections of subjects with low LBP (n=12) and subjects with high LBP (n=12) plasma levels. CD-68 staining in visceral fat samples showed that LBP was associated with degree of macrophage accumulation (low LBP group: 4.1±1.5 vs. high LBP group: 8.5±2.1 macrophages/cm², p<0.0001, Figure 1A).

In the total group, we also found a significant correlation between plasma LBP and amount of crown-like structures (Spearman’s rho=0.4, p<0.05)

Intestinal microbiota composition and diversity

No significant difference between the composition of the fecal communities of the low- and high LBP groups could be detected. However, there was a tendency that bacteria belonging to the Bacteroidetes phylum correlated with the high LBP group. Similary, the high LBP group tended to have a lower overall diversity. Of note, we found no association between intestinal microbiota composition and the amount of macrophages in visceral adipose tissue.
Role of microbiota in visceral adipose inflammation

Figure 1A. Macrophage infiltration in visceral adipose tissue

Figure 1B. Crown-like structures in visceral adipose tissue
Data are presented as median (range).
Bacterial DNA in visceral adipose tissue

Using DGGE gel, we identified a common bacterial DNA band in all visceral adipose tissue specimens (Figure 2). Upon cloning and subsequent sequencing, we identified bacteria related to the gram-negative *Ralstonia* species to be the ubiquitous bacterial species in visceral adipose tissue. By applying a PCR for quantification of *Ralstonia* species, the amount of *Ralstonia* DNA was significantly increased in subjects with low LBP levels compared to the high LBP group (low LBP group: 84.6±7.1 vs. high LBP group: 59.5±8.6 bacterial *Ralstonia*/total bacterial DNA, p<0.05, Figure 3).

Figure 2. Bacterial DNA in visceral adipose tissue using DGGE gel
The arrow indicates the dominant species.

Figure 3. Amount of bacterial DNA in the low vs. high LBP group
The horizontal line represents the mean value (SEM).
Discussion

In the present study we found that endotoxemia was associated with macrophage accumulation in visceral fat tissue of otherwise healthy obese subjects. Moreover, differences in intestinal microbiota composition were paralleled by the presence of *Ralstonia* DNA in visceral adipose tissue. Our data suggest that plasma endotoxemia could be a marker of chronic visceral adipose tissue inflammation in human obesity and several other observations point towards a role for bacteria related to the gram-negative *Ralstonia* in this process.

First, we confirmed that plasma LBP is associated with chronic inflammation in obese subjects (12). Obese subjects are characterized by enhanced bacterial translocation (33), but at the same time obesity seems to be propagated by bacterial endotoxins, such as lipopolysaccharides (LPS) derived from intestinal bacteria (e.g. *Proteobacteria*), which are found in relatively large numbers in the human intestine of obese subjects (34;35). Translocation of intestinal gram-negative bacteria can induce a local inflammatory response and subsequent macrophage influx in the visceral adipose tissue of obese mice, underscoring a role for the innate immune system and intestinal microbiota in chronic adipose tissue inflammation (36). Although in humans there is much debate whether intestinal microbiota composition is cause or mere consequence of obesity (15;22;23), invasion of visceral adipose tissue by pro-inflammatory macrophages is still considered to be a key event driving adipose-tissue inflammation and insulin resistance (37).

Second, our data are the first indication that macrophage influx in (visceral) adipose tissue might be elicited by translocation of gram-negative intestinal bacteria. Previous evidence in mice likewise showed an association between chronic inflammatory tone and bacterial translocation (20). Toll-like receptor 5 (TLR5) seems to play a pivotal role in preventing bacterial translocation in the intestine (38) and diet induced obesity was recently found to be associated with increased TLR5 mRNA expression as well as macrophage count in murine adipose tissue (39). As TLR5 is predominantly involved in the capture of gram-negative flagellin-bearing bacteria (which are subsequently
cleared by macrophages), the presence of DNA from *Ralstonia* (a flagellin-bearing member of the *Burkholderia* group) in human visceral adipose tissue corroborates with earlier findings in animal models. The *Ralstonia* genus (Proteobacteria phylum) comprises the Ralstonia eutropha (7 species) and Ralstonia pickettii (5 species) lineage that are closely related to *Burkholderia* spp. (40), bacteria that flourish in a high carbohydrate/glucose milieu (41). In this respect, it is interesting to note that another member of the *Burkholderia* group, *B. ambivaria*, uses small fucosylated glycans on intestinal epithelium to translocate into the bloodstream (42). Fucose is highly abundant in the intestine and it has been long recognized that obesity and insulin resistance increase fucose levels in humans (43). This could also explain the *Bacteroides*, which are known to harbour multiple fucosidases that cleave fucose from host glycans, resulting in high fucose availability in the intestinal lumen (44).

Our study has certain limitations. Firstly although we use LBP as an indirect plasma marker for endotoxemia, LBP has not been validated yet as predictive plasma marker of inflamed visceral adipose tissue in large prospective cohorts. Secondly, a potential bias could be the presence of cholecystolithiasis (or gallstones), being the indication of surgery for all subjects. As the relationship between the presence of specific intestinal microbiota and the development of gallstones is currently unknown, this might have imposed a bias. However, all patients had symptomatic cholecystolithiasis indicating similar disease severity. Finally, subjects with high endotoxemia were characterized by increased BMI. Although this could imply selection bias, endotoxemia was found to be associated with the development of obesity in humans (7;33).

In conclusion, we show that endotoxemia is associated with the level of macrophage accumulation in visceral fat tissue of otherwise healthy obese subjects. Moreover, as bacterial DNA of *Ralstonia* was found to be inversely related to the amount of visceral adipose tissue macrophages, our data imply that the intestinal microbiota may play an initiating role in chronic inflammation of visceral adipose tissue thus providing novel therapeutic targets.
Acknowledgments
We would like to thank N. Rossen MD (dept of gastroenterology, AMC) for help with the FISH. We would also express our gratitude to Ineke Heikamp-de Jong, Philippe Puylaert and Wilma Akkermans-van Vliet (Wageningen University) for excellent laboratory assistance.

Grant support

Competing financial interests
The authors declare no competing financial interests
Chapter 7

References

Role of microbiota in visceral adipose inflammation

Chapter 7


