Research Article

JOURNAL OF HEPATOLOGY

Gut-adipose tissue axis in hepatic fat accumulation in humans

Eveliina Munukka[1,2], Satu Pekkala[1], Petri Wiklund[1], Omid Rasool[3], Ronald Borra[4], Lingjia Kong[5], Xiaowei Ojanen[6], Shu Mei Cheng[1], Christophe Roos[5,†], Soile Tuomela[3], Markku Alen[7,8], Riitta Lahesmaa[3], Sulin Cheng[1,9,*]

[1]*Department of Health Sciences, University of Jyväskylä, P.O. Box 35, 40014 University of Jyväskylä, Finland;* [2]*Department of Medical Microbiology and Immunology, University of Turku, Finland;* [3]*Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Turku, Finland;* [4]*Department of Diagnostic Radiology, Turku University Hospital, Turku, Finland;* [5]*Tampere University of Technology, Tampere, Finland;* [6]*Department of Applied Physics, University of Eastern Finland, Kuopio, Finland;* [7]*Department of Medical Rehabilitation, Oulu University Hospital, Oulu, Finland;* [8]*Institute of Health Sciences, University of Oulu, Finland;* [9]*Department of Physical Education, Shanghai Jiao Tong University, Shanghai, China*

See Editorial, pages 5–6

Background & Aims: Recent evidence suggests that in animals gut microbiota composition (GMC) affects the onset and progression of hepatic fat accumulation. The aim of this study was to investigate in humans whether subjects with high hepatic fat content (HHFC) differ in their GMC from those with low hepatic fat content (LHFC), and whether these differences are associated with body composition, biomarkers and abdominal adipose tissue inflammation.

Methods: Hepatic fat content (HFC) was measured using proton magnetic resonance spectroscopy (^{1}H MRS). Fecal GMC was profiled by 16S rRNA fluorescence *in situ* hybridization and flow cytometry. Adipose tissue gene expression was analyzed using Affymetrix microarrays and quantitative PCR.

Results: The HHFC group had unfavorable GMC described by lower amount of *Faecalibacterium prausnitzii* (FPrau) (p <0.05) and relatively higher Enterobacteria than the LHFC group. Metabolically dysbiotic GMC associated with HOMA-IR and triglycerides (p <0.05 for both). Several inflammation-related adipose tissue genes were differentially expressed and correlated with HFC (p <0.05). In addition, the expression of certain genes correlated with GMC dysbiosis, i.e., low FPrau-to-Bacteroides ratio.

Conclusions: HHFC subjects differ unfavorably in their GMC from LHFC subjects. Adipose tissue inflammation may be an important link between GMC, metabolic disturbances, and hepatic fat accumulation.

Keywords: *Faecalibacterium prausnitzii*; Microbiota dysbiosis; Adipose tissue inflammation; Leaky gut.

Received 23 October 2013; received in revised form 27 January 2014; accepted 25 February 2014; available online 5 March 2014

☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2014.04.006.

* Corresponding author. Address: Department of Health Sciences, FI-40014 University of Jyväskylä, Finland. Tel.: +358 40 558 0209; fax: +358 14 260 2011. *E-mail address:* sulin.cheng@jyu.fi (S. Cheng).

† Present affiliation: Euformatics Oy, Espoo, Finland.

Abbreviations: Bacto group, *Bacteroides* group; FPrau, *Faecalibacterium prausnitzii*; FPrau-to-Bacto, *F. prausnitzii* to *Bacteroides* ratio; GM, gut microbiota; GMC, gut microbiota composition; HFA, hepatic fat accumulation; HFC, hepatic fat content; HHFC, high hepatic fat content group; HOMA, homeostasis assessment index; IR, insulin resistance; LHFC, low hepatic fat content group; LPS, lipopolysaccharide; MBI, microbial balance index; MRI, magnetic resonance imaging; ^{1}H MRS, proton magnetic resonance spectroscopy; NAFLD, non-alcoholic fatty liver disease.

Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) ranges from 20% to 30% in the general population up to 75% in obese individuals in western countries [1]. NAFLD has been predicted to be the main cause of liver transplantation by the year 2020 due to the ongoing worldwide epidemic of obesity and type 2 diabetes [2]. Nevertheless, the underlying mechanisms of NAFLD-related hepatic fat accumulation (HFA) remain poorly understood. One proposed mechanism is an excess free fatty acid (FFA)-induced lipotoxicity that sensitizes the liver to chronic inflammation [3]. In addition, the role of dysfunctional adipose tissue in the pathogenesis of HFA has been emphasized [4].

There is emerging evidence that gut microbiota composition (GMC) and functionality may also have a crucial role in the HFA and pathogenesis of NAFLD [5–7]. The crosstalk between liver and gut is likely since liver receives metabolite-rich blood *via* portal vein draining from the gut [8]. A disrupted gut epithelium that leads to hyperpermeability, i.e., leaky gut, may participate in the inflammation associated with HFA by enabling bacterial translocation from the gut [8,9]. However, the preliminary results concerning crosstalk between GMC and liver are mainly derived from rodent studies. Data obtained in humans remains scarce [5–9].

This cross-sectional human study aimed to investigate whether subjects with high and low hepatic fat content (HFC) differ in their GMC and whether the differences are associated with body composition and adipose tissue inflammation.

Patients and methods

Subjects

Participants (42–63 years old) were recruited from a larger study (AMB-study) approved by the ethics committee of the Central Finland Health Care district. Exclusion criteria were: severe cardiovascular, musculoskeletal diseases or chronic gastrointestinal problems, extreme diets, or antibiotic course (<3 months prior study).

Altogether 87 participants enrolled to the study. Only subjects over 40 years (n = 59) were included in the analysis, and from them 33 had both MRI scan and fecal sample. One subject was excluded due to celiac disease and one due to high daily consumption of alcohol. Thus the final subjects included in this report were 31. Informed consent was obtained from subjects prior the study.

Each subjects' characteristics are presented in Supplementary Table 1. Medical history, health status, and lifestyle information were collected *via* self-administered questionnaires. Total energy intake was recorded using three-day food diaries and analyzed with Micro-Nutrica software [10]. Subjects were classified into high hepatic fat content group (HFC >5%, n = 10) or low hepatic fat content group (HFC ⩽5%, n = 21) based on *in vivo* proton magnetic resonance spectroscopy assessment (^{1}H MRS) (Supplementary Table 1).

Anthropometrics and body composition

Anthropometrics (height, weight, waist circumference, BMI) were measured as described earlier [11]. Body composition was assessed with dual-energy X-ray absorptiometry (DXA, Prodigy; GE Lunar Corp, Madison, WI, USA). Precision of the repeated measurements expressed coefficient of variation (CV) 2.2% for fat mass (FM) and for lean mass (LM) 1.0%.

Hepatic fat content

The assessment of HFC (%) was performed by ^{1}H MRS (1.5T GE Signa CV/i scanner, GE Medical Systems, Waukesha, WI, USA) [12]. Spectra were analyzed using Linear Combination of Model spectra software package (LCModel version 6.1-4, LCMgui user interface version 2.1-4), which is considered as golden standard for *in vivo* spectroscopy analysis [13].

Abdominal fat assessment

Visceral subcutaneous and retroperitoneal fat were segmented using a single slice image at the level of the L2-L3 intervertebral disc using the OsiriX software (OsiriX Foundation, Geneva, Switzerland). The level of the single-slice visceral fat measurement has been shown to correlate with the total abdominal visceral fat mass (R = 0.95 and 0.97) in both sexes [14].

Fecal samples

Fecal samples were frozen and stored at −80 °C. GMC was profiled with a previously described method using 16S rRNA hybridization, DNA-staining and flow cytometry (BD FACSCalibur™, Becton Dickinson, San Jose, CA, USA) [15]. Six 16S rRNA-targeted oligonucleotide probes were used: Ato291 for the *Atopobium* cluster, Bacto1080 for the *Bacteroides* group, Bif164 for *Bifidobacterium* sp, Enter1432 for enteric group bacteria, Erec482 for the *Clostridium cluster* XIVa, and Fprau645 for *Faecalibacterium prausnitzii* [15–17].

From the bacterial groups, three GMC indexes were computed: The *F. prausnitzii* to *Bacteroides* (Fprau-to-Bacto) ratio and *Firmicutes*-to-*Bacteroidetes* ratio (F/B ratio) both illustrating metabolic status of GMC, and Microbial Balance Index (MBI) the division of the sum of bifidobacteria and FPrau by the sum of the Bacto and the enteric group describing the overall balance of GM [16].

Blood samples

Serum and plasma from fasting blood samples were stored at −80 °C. Plasma glucose and serum triglycerides, total cholesterol, high density lipoprotein cholesterol (HDL), and activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analysed using KONELAB 20XTi analyzer (Thermo Fischer Scientific Inc., Waltham, MA, USA). Insulin was determined using IMMULITE Analyser (Diagnostic Products Corporation, Los Angeles). LDL cholesterol was calculated using the Friedewald equation. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as (insulin concentration x glucose concentration)/22.5.

Serum leptin was assessed using ELISA (DuoSet®, R&D Systems, MN, USA). Plasma LPS concentration was detected by limulus assay (LAL Chromogenic Endpoint Assay, HyCult biotech, PA, USA).

Adipose tissue RNA isolation and microarray experiments

A needle biopsy (14 G, ø 2.1 × 60 mm) of subcutaneous abdominal adipose tissue was taken after overnight fasting, under local anesthesia. Samples were frozen in liquid nitrogen and stored at −80 °C.

Total RNA was extracted from biopsies using the FastPrep system (MP Biomedicals, France) and the RNeasy Lipid Tissue Mini Kit (QIAGEN, Gaithersburg, MD, USA) according to manufacturer's instructions. Total RNA was digested on-column with the RNase-free DNase set (QIAGEN) during RNA isolation. The quality of the total RNA was studied using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and a Experion Automated Electrophoresis Station (BioRad, Hercules, CA, USA). The total RNA was amplified and processed using the GeneChip 3IVT Express Kit (Affymetrix, Santa Clara, CA, USA) and hybridized on Affymetrix Human Genome U219 Array Plates.

Quantitative PCR

Microarray results were confirmed by qPCR of *MMP9* and *VAV1* from the same RNA samples. High Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe 230 ng of RNA. Real-time PCR analysis was performed using iQ SYBR Supermix and CFX96™ Real-time PCR Detection System (Bio-Rad Laboratories, Richmond, CA, USA).

The primer sequences were as follows:

MMP9 sense: 5′-GAGTGGCAGGGGGAAGATGC-3′, and antisense 5′-CCTCAGGG CACTGCAGGATG-3′

VAV1 sense: 5′-AGCAGTGGGAAGCACAAAGTATT-3′, and antisense 5′-GTCAC GGGCGCAGAAGTC-3′

GAPDH sense: 5′-CCACCCATGGCAAATTCC-3′ and antisense: 5′-TGGGATTTCC-ATTGATGACAA-3′

Relative expression levels for *MMP9* and *VAV1* were calculated with the $\Delta\Delta C_t$ method and normalized to the expression of *GAPDH*. The fold changes of each gene between the groups were similar to those detected in the microarray analysis (data not shown).

Statistical analyses

Data were checked for normality using Shapiro-Wilk W test (IBM SPSS Statistics 19). If data were not normally distributed, natural logarithms were used. Descriptive results are given as means and standard deviation (SD). Student's paired *t* test was used for comparisons of the blood and fecal analyses results, in addition to control the potential confounders, analysis of covariance (ANCOVA) was performed to control for age, gender, and weight. Partial Spearman correlation adjusted for age, gender, and weight was calculated for GMC parameters with HFC (%) and certain biochemical markers. Statistical significance was set at p <0.05.

Microarray measurements were analyzed by using the Robust Multiarray Averaging (RMA) algorithm in the Bioconductor R package affy [18–21]. The Limma R package was used for differentially expressed genes (DEGs) [22]. Raw p values were adjusted to control for the false discovery rate (FDR) using the method of Benjamini and Hochberg [23].

Results

Study population

The HHFC subjects were older, heavier, had greater WC, higher total and visceral FM compared to the LHFC subjects (p <0.05 for all, Table 1). No differences were found in height, BMI, LM, FM% or total daily energy intakes between the groups (p >0.05 for all, Table 1).

The HHFC subjects had higher glucose, insulin, HOMA-IR, triglycerides and leptin than the LHFC subjects (p <0.05 for all,