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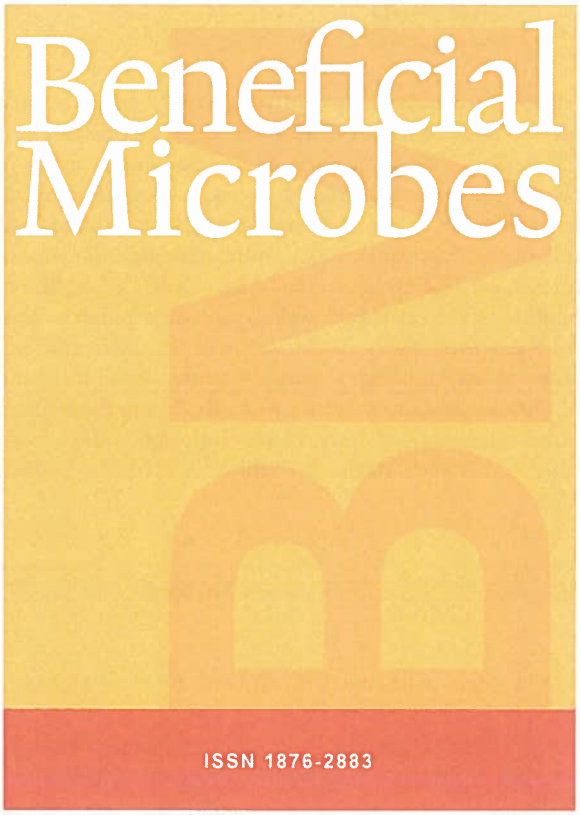

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Gut microbiota composition strongly correlates to peripheral insulin sensitivity in obese men but not in women

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RESEARCH ARTICLE

Abstract

Gut microbiota composition may play an important role in the development of obesity-related comorbidities. However, only few studies have investigated gender-differences in microbiota composition and gender-specific associations between microbiota or microbial products and insulin sensitivity. Insulin sensitivity (hyperinsulinemic-euglycemic clamp), body composition (dual energy X-ray absorptiometry), substrate oxidation (indirect calorimetry), systemic inflammatory markers and microbiota composition (PCR) were determined in male (n=15) and female (n=14) overweight and obese subjects. *Bacteroidetes/Firmicutes*-ratio was higher in men than in women (P=0.001). *Bacteroidetes/Firmicutes*-ratio was inversely related to peripheral insulin sensitivity only in men (men: P=0.003, women: P=0.882). This association between *Bacteroidetes/Firmicutes*-ratio and peripheral insulin sensitivity did not change after adjustment for dietary fibre and saturated fat intake, body composition, fat oxidation and markers of inflammation. *Bacteroidetes/Firmicutes*-ratio was not associated with hepatic insulin sensitivity. Men and women differ in microbiota composition and its impact on insulin sensitivity, implying that women might be less sensitive to gut microbiota-induced metabolic aberrations than men. This trial was registered at clinicaltrials.gov as NCT02381145.

Keywords: insulin sensitivity, *Bacteroidetes*, *Firmicutes*, obesity, fat oxidation, gender

1. Introduction

Gut microbiota dysbiosis has been associated with metabolic impairments, such as dyslipidemia and reduced insulin sensitivity in obese, non-diabetic subjects (Le Chatelier *et al.*, 2013). In obese humans, lower ratios of the two dominant phyla of intestinal bacteria, *Bacteroidetes* and *Firmicutes*, have been found (Ley *et al.*, 2006; Schwiertz *et al.*, 2010; Turnbaugh *et al.*, 2009; Verdam *et al.*, 2013), yet a recent meta-analysis has found no significant association between the ratio (or individual phyla) and obesity status (Sze and Schloss, 2016).

Contrary to the relation with obesity, the *Bacteroidetes/Firmicutes*-ratio has been reported to be associated with elevated glucose concentrations after an overnight fast and during an oral glucose tolerance test in diagnosed diabetic patients as compared to healthy volunteers in some (Larsen *et al.*, 2010; Remely *et al.*, 2013), but not all studies (Zhang *et al.*, 2013). The glucometabolic status should therefore be considered when developing strategies to control metabolic diseases such as obesity by modifying the gut microbiota.

In more recent studies (Reijnders *et al.*, 2016; Vrieze *et al.*, 2012, 2014), detailed characterisation of tissue-specific insulin sensitivity by means of hyperinsulinemic-euglycemic clamp has been performed before and after modulation of microbial composition by means of antibiotics-intervention or after faecal transplantation in subjects with the metabolic syndrome. To distinguish between hepatic and peripheral insulin sensitivity may provide new insight in the relationship between the gut microbiota and metabolic health because both may be affected through different metabolic pathways (Scheithauer *et al.*, 2016). In fact, the

former study showed that microbial composition changes affected had more pronounced effects on peripheral as compared to hepatic insulin sensitivity (Vrieze *et al.*, 2014). In addition, gender differences have not been taken into account in these studies. Noteworthy, the susceptibility to obesity and related co-morbidities after induction of microbial dysbiosis may be different in males and females (Cox *et al.*, 2014; Murphy *et al.*, 2014). For example, a significant interaction between early life antibiotic use and childhood body mass index (BMI) has been found for boys, but not for girls (Murphy *et al.*, 2014), and alterations in the microbiota led to more pronounced increases in hepatic steatosis in males as compared to females (Cox *et al.*, 2014).

Here, we analysed cross-sectional data on gender-related differences in microbiota composition and its relation with host metabolic phenotype, including tissue-specific insulin sensitivity, substrate oxidation and body composition in overweight and obese, non-diabetic Caucasian men (n=15) and women (n=14).

2. Materials and methods

Analysed data were baseline measurements from subjects that were recruited for a 12-week polyphenol-supplementation study (Most *et al.*, 2016), in which effects on insulin sensitivity (primary outcome), substrate oxidation and microbiota composition (secondary outcomes) were assessed. This ancillary paper will not discuss the primary outcomes of the polyphenol-intervention study, but will focus on the association between gender, microbiota composition and insulin sensitivity. Participants were recruited within the vicinity of the study centre at Maastricht University, were sedentary (<3 h/week in sports activities) and had no cardiometabolic or gastrointestinal complications or diseases. Subjects had been weight-stable and completed a detailed medical and lifestyle questionnaire, had not taken antibiotics or medication that may interfere with study outcomes for at least three months prior to enrollment.

We assessed insulin sensitivity by combining a two-step hyperinsulinemic-euglycemic clamp with stable-isotope [6,6-$^2H_2$]-glucose tracer-infusion (Cambridge Isotope Laboratories, Andover, MA, USA). This enabled determination of peripheral and hepatic insulin sensitivity, expressed as insulin-stimulated rate of disappearance (Rd, μM/(kg×min)) at an infusion-rate of 40 mU/(m^2×min) and suppression of endogenous glucose production (suppression EGP, %) at 10 mU/(m^2×min), respectively.

Faecal samples were collected at the study centre during one of the three visits before the intervention (Most *et al.*, 2016). Samples were scooped into collection vials and immediately frozen in liquid nitrogen at -80 °C until further analysis. For enumeration of selected bacterial groups, metagenomic DNA was extracted and subjected to PCR assays targeting the major bacterial phyla (Applied Biosystems, Waltham, MA, USA). To extract DNA, approximately 200 mg faeces was added to a 2 ml vial containing approximately 500 mg of zirconia beads (0.1 mm) and 4 glass beads (3 mm) and 1.2 ml of lysis buffer P from the PSP SPIN Stool DNA kit (Stractec Biomedical, Berlin, Germany). Samples were treated in a Magna Lyser at 5.5 ms in 3 cycles of 1 min and cooled on ice in between cycli. Subsequently, the DNA isolation was continued using the PSP SPIN Stool DNA plus kit as per the manufacturer's instructions and finally eluted in 200 μl. For γ-*Proteobacteria*, *Actinobacteria*, *Akkermansia muciniphila* (phylum *Verrucomicrobia*) and *Bacteroidetes*, real-time detection of PCR-products was conducted with SYBR Green I (Bio-rad Laboratories Inc., Hercules, CA, USA) (Bacchetti de Gregoris *et al.*, 2011; Collado *et al.*, 2007). For *Firmicutes*, the 5'-nuclease technique was used (ABgene, Hamburg, Germany) (Armougom *et al.*, 2009). Log_{10} DNA copies for a given microbial group/species per gram of wet weight faeces were calculated for each stool sample from the Ct-values using standard curves that were constructed using serial diluted (10^1-10^7 copies/PCR) recombinant plasmid constructs containing the amplicon of interest.

Dietary intake of macronutrient intake and saturated fat (all expressed as percentage of energy intake, %EI) and fibre (g/MJ) were calculated from 3-day food records. An experienced dietitian checked the food records and discussed these with the subjects in case of incomplete or missing information. Energy and nutrient intakes were analysed by using the Dutch Food Composition dataset (NEVO; National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands). In addition, standardised meals were provided for the evenings prior to days of clinical assessments and sample collection. Body fat percentage was determined by dual energy X-ray absorptiometry (DXA, Hologic BCA; VitaK, Maastricht, the Netherlands) and waist and hip circumferences were measured using a non-expandable measuring tape midway between the lower rib margin and the iliac crest ('waist'), at largest circumference between the waist and the thighs ('hip') respectively. Energy expenditure (EE) and fat oxidation (expressed as percentage of EE, %EE) was determined by means of indirect calorimetry (Omnical; Maastricht University, Maastricht, the Netherlands) in a half-supine position during fasting conditions and for 4 h postprandially (2.6 MJ, 61 energy% fat) (Most *et al.*, 2016). Fasting blood samples were taken for measurements of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) using a multiplex ELISA (Human ProInflammatory II 4-Plex Ultra-Sensitive Kit; Meso Scale Diagnositics, Rockville, MD, USA).

Gender differences were analysed by Students' unpaired *t*-test. All variables were tested for outliers and normal distribution (Shapiro-Wilk). We performed regression

analyses gender-specifically with insulin sensitivity as dependent and bacterial groups as independent variables, corrected for age (Model 1). Statistically significant relations were then subsequently adjusted for putative relevant covariates. Independent variables were included for food intake (FI, dietary saturated fat and fibre, Model 1+FI), body composition (BC, body fat percentage and distribution, Model 1+BC), fat oxidation (FO, fasting and postprandial, Model 1+FO) and systemic inflammatory markers (SIM, TNF-α and IL-6, Model 1+SIM). TNF-α was ln-transformed, because it was not normally distributed. Independent variables showed no inter-correlations (defined as r>0.80).

All subjects gave written informed consent before participation in this study, which was reviewed and approved by the local Medical Ethical Committee of Maastricht University Medical Centre+. All procedures were performed according to the declaration of Helsinki.

3. Results

We found higher copy numbers of γ-*Proteobacteria* and *Bacteroidetes* in men than women, while presence of other phyla were not different between gender (Table 1). Furthermore, the *Bacteriodetes*/*Firmicutes* (B/F)-ratio was significantly higher in men as compared to women (P<0.001, Table 1).

Interestingly, a significant inverse linear association was found between the B/F-ratio and peripheral insulin sensitivity in men (P=0.003; Figure 1A) but not in women (P=0.882; Figure 1B). No significant correlations were found between B/F-ratio and hepatic insulin sensitivity for both gender (Figure 1C-D). Specific phyla copy numbers (γ-*Proteobacteria*, *Actinobacteria*, *A. muciniphila*, *Bacteroidetes* and *Firmicutes*) were not significantly correlated with any measure of insulin sensitivity in men or women (data not shown).

Macronutrient composition, dietary fibre and saturated fat intake (relative to energy intake) were comparable for men and women, while body fat percentage and distribution were different, as expected (Table 1). Of note, we found that food intake was not significantly altered throughout the 12 week-intervention that was initiated after the baseline assessments (data not shown). Based on this we are confident that food intake was similar before the intervention and well represented by the food intake records we collected. While fasting fat oxidation (%energy expenditure, EE) and IL-6 were comparable between gender, postprandial fat oxidation (%EE) and TNF-α were higher in men vs women (Table 1). Further adjustment for dietary fibre and saturated fat intake (Model 1+FI), body fat percentage and distribution (Model 1+BC), fasting and postprandial fat oxidation (Model 1+FO) and plasma inflammatory markers, TNF-α and IL-6 (Model 1+SIM) did not change the relation between B/F-ratio and peripheral insulin sensitivity in men, as indicated by similar standardised beta-coefficients for the B/F-ratio in the models (Model 1: Std.β(B/F-ratio)=-0.71, P=0.005; Model 1+FI: -0.70, 0.011; Model 1+BC: -0.79, 0.004; Model 1+FO: -0.65, 0.013; Model 1+SIM: -0.75, 0.005).

4. Discussion

The *Bacteroidetes*-content and the B/F-ratio were significantly higher in men as compared to women, which is in line with previous findings (Dominianni *et al.*, 2015). Importantly, differences in ethnicity (Caucasian), age (20-50 years), BMI (27-40 kg/m^2) and regular exercise (<3 h/week) cannot explain these discrepancies, because groups were well-matched for these parameters in the present study. However, early life acquisition (delivery mode, breastfeeding status, use of antibiotics), lifestyle (local environment, eating and defecation pattern, exercise) and the hormonal environment may underlie the gender-specific microbiota composition.

Using the gold-standard hyperinsulinemic-euglycemic clamp method we found an inverse association of B/F-ratio with peripheral insulin sensitivity, in men but not in women. Thus, the impact of microbiota on tissue-specific insulin sensitivity seems to differ between gender. Indeed, there is evidence for an increased gender-specific susceptibility to obesity and related co-morbidities after induction of microbial dysbiosis (Cox *et al.*, 2014; Murphy *et al.*, 2014). While no such information is available for adult humans yet, childhood obesity was significantly more associated to early life antibiotic exposure in boys than girls (5-8 years) after adjustment for several cofactors (Murphy *et al.*, 2014). Likewise, the combination of antibiotic-treatment and high-fat diet, independently and synergistically, evoked alterations in microbial composition, increased obesity and hepatic steatosis, with all these effects being more pronounced in male than female mice (Cox *et al.*, 2014). Of note, this detrimental phenotype was also observed after transfer of faecal microbiota from antibiotic-treated animals to germ-free mice, eliminating direct effects of antibiotic treatment itself. Finally, also gender-specific effects of diet on vertebrate's microbiota have been previously reported (Bolnick *et al.*, 2014). Therefore, it is tempting to speculate that our gut microbiota may be involved as a determinant in differential gender-related metabolic responses to dietary interventions.

The inverse relationship between B/F-ratio and peripheral insulin sensitivity in men is consistent with a relative higher abundance of *Bacteroidetes* and deteriorated metabolic profile in non-diabetic subjects (Le Chatelier *et al.*, 2013). Likewise, a reduction in *Firmicutes* and decreased peripheral insulin sensitivity was observed after antibiotic-treatment in obese men with the metabolic syndrome

Table 1. Subject characteristics (mean ± standard deviation).

Subject characteristics[1]		Men (n=15)	Women (n=14)	*P*-value[2]
	Age, yrs	39.2±2.4	35.1±3.0	0.291
	BMI, kg/m^2	29.4±0.7	29.4±0.7	0.992
Insulin sensitivity	Rd, µM/(kg×min)	29.6±2.4	35.6±3.2	0.142
	EGP suppression, %	46.5±5.4	68.4±3.3	0.002
Food intake	Fibre, g/MJ	1.8±0.1	1.9±0.2	0.741
	SFA, %EI	17.9±0.9	16.8±0.8	0.370
	Carbohydrate, %EI	43.9±1.6	46.5±1.4	0.239
	Protein, %EI	15.3±0.8	15.4±0.8	0.981
	Fat, %EI	38.9±1.8	36.0±1.3	0.226
Body composition	Body fat, %	23.4±0.8	37.0±1.0	<0.001
	WHR	0.92±0.02	0.79±0.01	<0.001
	Visceral fat, g	500±45	387±42	0.080
Fat oxidation	Fasting, %EE	56.1±2.8	54.7±3.8	0.766
	Postprandial, %EE	52.5±2.7	45.3±2.5	0.057
Systemic inflammation	TNF-α, pg/ml	3.49±0.22	2.28±0.09	<0.001
	IL-6, pg/ml	0.73±0.14	0.93±0.17	0.373
Bacterial composition	γ-Proteobacteria, log	11.3±0.1	10.9±0.1	0.028
	Actinobacteria, log	11.5±0.1	11.7±0.1	0.345
	Akkermansia muciniphila, log	8.7±0.5	9.6±0.4	0.186
	Firmicutes, log	12.3±0.1	12.4±0.1	0.402
	Bacteroidetes, log	13.2±0.1	12.8±0.1	0.001
	B/F-ratio	10.13±1.66	3.15±0.48	0.001

[1] BMI = body mass index; Rd = insulin-stimulated glucose rate of disappearance; EGP = insulin-mediated suppression of endogenous glucose production; SFA = saturated fatty acids; EI = total daily energy intake; WHR = waist-to-hip ratio; EE = energy expenditure; TNF-α = tumour necrosis factor-alpha; IL-6 = interleukin 6; B/F-ratio = faecal *Bacteroidetes*/*Firmicutes*-ratio

[2] Statistical significance of Student's unpaired t-test.

(Vrieze *et al.*, 2014). Interestingly, in accordance with our findings, induced alterations in gut microbiota composition were paralleled by a reduction in peripheral, but not hepatic insulin sensitivity (Vrieze *et al.*, 2014).

Several lifestyle and metabolic factors may mediate the relationship between B/F-ratio and insulin sensitivity (Khan *et al.*, 2014). Dietary fibre intake has been associated with beneficial effects on glucose metabolism, while high-fat diets were associated with reduced insulin sensitivity. A reduced bacterial diversity and altered microbial composition, induced by high-fat, low-fibre diets, may contribute to disturbances in substrate oxidation and insulin sensitivity by modulation of short-chain fatty acid (SCFA) and bile acid concentrations in the gut and circulation. Additionally, our microbiome may affect insulin sensitivity through induction of inflammatory and hormonal signals (lipopolysaccharides, respectively glucagon-like-peptide 1). Taken together, there appears to be a strong interaction between dietary factors, the microbial composition and impaired insulin sensitivity. Therefore, we investigated whether the relationship between the B/F-ratio and insulin sensitivity in men can, at least partly, be explained by lifestyle factors such as dietary fibre and saturated fat intake, or metabolic factors such as fat oxidation and low-grade inflammation. Strikingly, adjustment for the above-mentioned factors did not change the strong association between the B/F-ratio and peripheral insulin sensitivity in men, suggesting involvement of other factors.

It is beyond the scope of this study, which is associative in nature, to draw any conclusions on putative mechanisms responsible for the strong association between B/F-ratio and peripheral insulin sensitivity. However, our data clearly indicate that microbiome-host interactions should be taken into account in controlled intervention studies.

In conclusion, the present study demonstrated a significant difference in microbiota composition between men and women. Furthermore, we found a strong relationship between intestinal microbiota composition and peripheral, but not hepatic, insulin sensitivity in men but not in women, which remained unchanged after adjustment of dietary saturated fat and fibre intake, body composition,

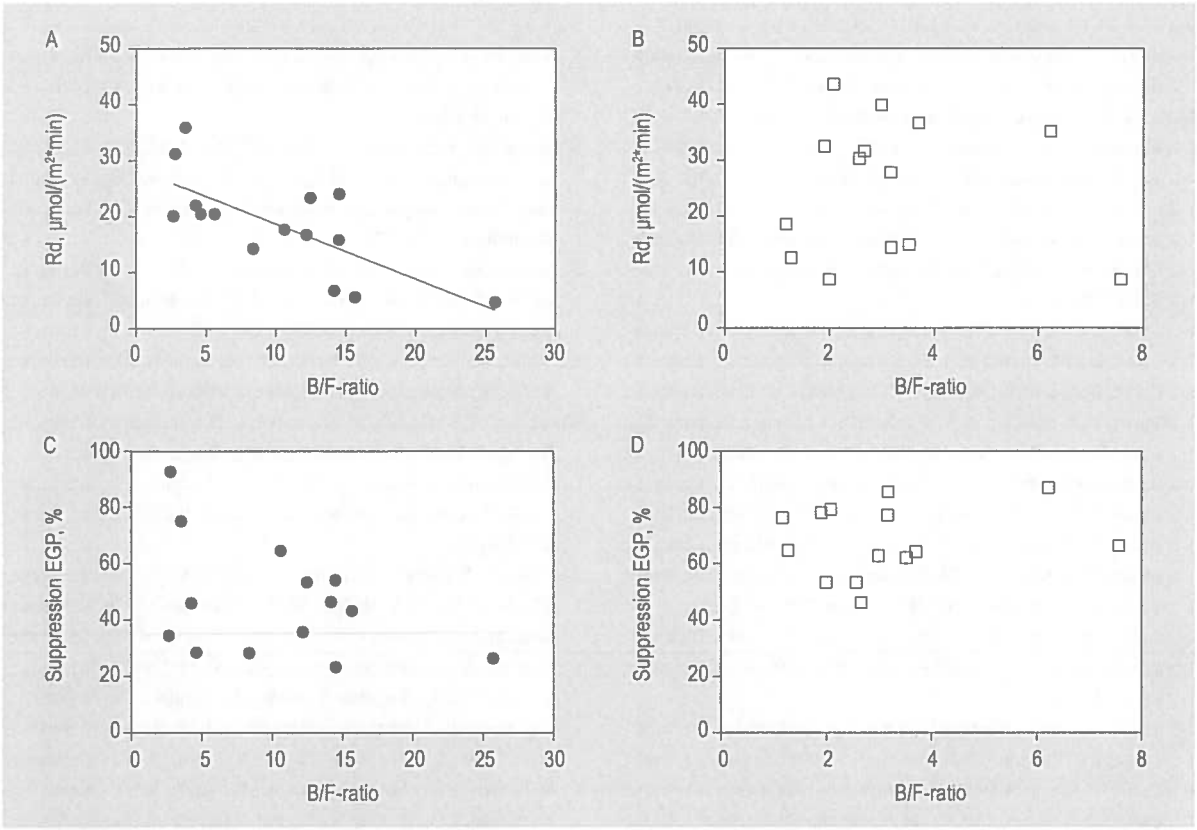

Figure 1. Relations between *Bacteroidetes/Firmicutes*-ratio (B/F-ratio) and insulin sensitivity by gender. B/F-ratio plotted against peripheral (A, B) and hepatic (C, D) insulin sensitivity of men (A, C) and women (B, D). Rd = insulin-stimulated glucose rate of disappearance; suppression EGP = insulin-mediated suppression of endogenous glucose production.

fat oxidation and systemic inflammation. Our findings are preliminary in nature and require confirmation in larger populations using state-of-the-art next generation sequencing. Understanding the aetiology of microbial dysbiosis, and elucidating how the microbiota impacts host metabolism in a gender-specific manner may open new avenues for the treatment of obesity-related cardiometabolic disorders.

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