Article

Influence of Chitosan treatment in obese subjects on surrogate plasma markers of cholesterol metabolism

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Abstract: Chitosan treatment results in significantly lower plasma LDL-cholesterol concentrations. To test the working mechanism of Chitosan, we measured plasma surrogate markers of cholesterol absorption, synthesis, and degradation to bile acids corrected for cholesterol concentration (R_sterols). One hundred sixteen obese subjects (BMI 31.7, range 28.1 – 38.9 kg/m^2) were studied under Chitosan treatment (n=61) and placebo treatment (n=55) during 12 weeks. The participants underwent a short nutrition education on how to improve quality of nutrition and energy expenditure. Daily Chitosan intake was 3200 mg. RESULTS. Plasma LDL-cholesterol concentration decreased significantly stronger (P=0.0252) under Chitosan (-8.67 ± 18.18 mg/dl, 5.6%) than under placebo treatment (-1.00 ± 24.22 mg/dl, 0.9%). This reduction was not associated with corresponding decreases of markers of cholesterol absorption under Chitosan treatment. As a marker for cholesterol synthesis R_lathosterol showed a trend towards a stronger decrease under Chitosan treatment (P=0.0759). Regarding markers of bile acid synthesis, R_7α-hydroxy-cholesterol decreased significantly only under Chitosan treatment, but not stronger than under placebo treatment. In conclusion, a significant selective reduction of plasma LDL-cholesterol under Chitosan treatment is neither associated with an expected reduction of plasma surrogate markers of cholesterol absorption nor with expected increases of markers for cholesterol and bile acid synthesis.

Keywords: cholesterol synthesis; bile acid synthesis; cholesterol absorption; lathosterol; plant sterols; oxysterols; lipoproteins; lipid lowering; phytosterols; placebo

1. Introduction

Reports on Chitosan treatment in obese subjects have shown contradictory results on the reduction of weight and plasma lipids [1-3]. Results appear to be dependent on many factors such as the Chitosan product composition (percent of deacetylation and content of vitamin C and tartaric acid) and dosage as well as time of duration of treatment, group size, degree of obesity of subjects and accompanying weight reduction program. The intention for treatment with Chitosan is the binding of fat, cholesterol and bile acids in the stomach and intestine followed by increased fecal excretion of fat and cholesterol metabolites. Plasma total cholesterol is not reduced in all human studies [4-6], but in Cochran analysis [1] a small significant effect in favor of Chitosan was established (-0.15 mmol/L (95% CI -0.23 to -0.07)). LDL-cholesterol is commonly but not always [4,6] reduced under Chitosan treatment and a small but

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significant effect in favor of Chitosan was established (-0.16 mmol/L (95% CI -0.23 to -0.10)) in one study [1], which was the opposite in another study [7]. In animal studies large increasing effects of Chitosan on plasma HDL-cholesterol have been demonstrated [2] but the effect in humans appears significantly but marginally [1]: 0.03 mmol/L (95% CI 0.01 to 0.05). However, in many studies no considerable effects were shown [4,8-13]. The mechanism of action of Chitosan has not been fully understood to date. Chitosan is a soluble fiber that consists of polyglucosamine produced by deacetylation of Chitin. The amino groups are protonated in acidic environment. These hydrogen cations are able to bind to carboxylic compounds like fatty acids and bile acids. The major site of action is assumed to take place in the stomach where protonation is maximal favored by hydrogenium production from the goblet cells. However, prior to binding of free fatty acids, dietary triglycerides and phospholipids must be hydrolyzed by gastric lipase to free acids. Cholesterol and esterified cholesterol are not available in negatively ionized form. Under normal conditions bile acids are not present in the stomach.

A further effect of Chitosan is gel formation in the stomach [14-16]. Due to the high viscosity of the gastric content gastric emptying is delayed and rapid satiety is established [17]. With gradually increasing pH in the intestine and reduced ionic binding capacity of Chitosan , the gel transforms into a precipitate. It is assumed that the gel and the precipitate trap lipids and bile acids leading to increased fecal loss, however, in humans increased fat excretion was not confirmed in all studies [18]. Increased fecal cholesterol excretion has not been shown at all in humans. Fecal cholesterol excretion was not increased in the study of Maezaki et al [19]. In mice van Bennekum et al [17] did not find increased fecal excretion neither of cholesterol nor of bile acids under Chitosan treatment. In addition, no reduction of fractional cholesterol absorption rate was found. In contrast, they found a decreased food intake under Chitosan treatment. In rats Chitosan did lead to increased fecal excretion of cholesterol and bile acids [16]. However, Fukada et al [20] showed that Chitosan affected bacterial bile acid metabolism in rats but the quantitative bile acid excretion remained unchanged. In humans, the composition of fecal bile acids changed towards increased proportions of primary bile acids, but the total bile acid excretion rate did not change [19]. Thus, the working mechanism of Chitosan is not clear to date, in particular not in humans. Based on the expected increased fecal excretion of cholesterol metabolites and bile acids, it may be hypothesized that the observed reduction of plasma LDL-cholesterol is accompanied by reduced cholesterol absorption and increased cholesterol and bile acid synthesis.

Therefore, to investigate the mechanism of action for the hypocholesterolemic effect of Chitosan in humans, we studied the effect of Chitosan treatment on plasma markers of cholesterol absorption (campesterol, sitosterol, cholestanol), cholesterol synthesis (lathosterol, lanosterol, desmosterol), and bile acid synthesis (7α-hydroxycholesterol, 27-hydroxycholesterol) in obese volunteers.

2. Materials and methods

2.1. Study Design and Population

This study was part of a larger clinical trial designed as a 12-week, single center, randomized, placebo-controlled, double-blind, and parallel group study. The protocol was carried out with methods according to the guidelines for Good Clinical Practice (GCP) and the Declaration of Helsinki. It was approved by the Ethics Commission of the University Clinics Bonn (111/13-AMG-ff). Written informed consent was obtained from all participants. The clinical trial with a food supplement was registered at the European Clinical trials database (EudraCT number 2012-005475-13).

The study was performed at the phase I study unit of the Study Center Bonn (Head: Dr. med. C. Coch), Institute of Clinical Chemistry and Clinical Pharmacology (Head: Prof. Dr. med. G. Hartmann), University Clinics Bonn, Germany. The trial participants were recruited through advertisements in a daily newspaper, via wall posters presented at the wards as well as information at the University Clinics Bonn

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intranet. No dependent individuals were included in this trial. Main inclusion and exclusion criteria for this study were as follows. The subjects had to be aged 18-65 years having a BMI between 28 and 36 kg/m^2 at the time of presentation as well as a waist circumference of >88 cm (women) and >102 cm (men). Absence of relevant diseases was documented, e.g. absence of cardiovascular, hepatobiliary and gastrointestinal, previous or active malignant, neurological or psychiatric diseases or conditions after surgery. Excluded were Diabetes Mellitus type 1 and 2 patients, subjects with actual or suspected alcohol or drug abuse, subjects with weight reduction >5 kg within the last 5 months and subjects known to be allergic against crustaceans. Women at child bearing age had to show a negative pregnancy test and provide evidence of proper use of contraceptives or other factors excluding pregnancy to occur during the study. Subjects were not allowed to participate in other clinical trials.

After stratification according to their gender, patients were assigned to the respective groups using appropriateblock randomization. The chief investigator, investigators, study staff, bioanalytics and patients were all blinded to the treatment allocation in accordance with the double-blind design.

Participants in the Chitosan group (n = 61) received eight Chitosan containing tablets (Biopolymer3200, Certmedica International GmbH, Aschaffenburg, Germany), which were taken twice a day as 4 tablets with the main meal. Biopolymer3200 tablets consist of (ß-1,4-Polymer of D-Glucosamine and N-Acetyl-D-Glucosamine containing >80% Chitosan, 5-10% vitamin C, 1-5% tartaric acid and 5-10% water.

The participants in placebo group were given eight placebo tablets divided over two meals which contained 122.50 mg of microcrystalline cellulose, 372.50 mg of calcium hydrogen phosphate, 5.00 mg of magnesium stearate, 0.750 mg of iron oxide, yellow, 0.375 mg of iron oxide, brown, 0.375 mg of iron oxide black per tablet. During the study (9 visits follow-up), the remaining tablets were counted to check compliance. Participants also received nutritional advices. However, compliance to improved nutrition was not strictly monitored.

2.2. Blood sampling and sterol analysis

Fasting blood samples were collected before and after 12 weeks treatment. Plasma concentrations of total, HDL- and LDL-cholesterol were measured enzymatically by routine methods in the central laboratory of the university clinics of Bonn. The plasma concentrations of the surrogate markers of cholesterol absorption (campesterol, sitosterol, cholestanol), cholesterol synthesis (lathosterol, lanosterol, desmosterol) and bile acid synthesis (7α-hydroxy-cholesterol, 27-hydroxy-cholesterol) were measured with gas chromatography-mass spectrometry-selected ion monitoring [21,22]. In order to correct these markers for total cholesterol from the same sample we measured total cholesterol by gas chromatography-flame ionization detection [23]. These ratios indicated as R_sterols/oxysterols were used as markers of cholesterol absorption, synthesis and catabolism (= bile acid synthesis).

2.3. Statistics

The changes initiated by Chitosan and placebo treatment were tested against baseline using the two-tailed Wilcoxon test. The changes under Chitosan treatment and placebo treatment were compared using the two-tailed Mann-Whitney U test. This was done for the total group as well as for the groups of subjects experiencing an increase or decrease. The frequencies of treatment response were tested with the Fisher´s exact test. The correlation between the change of parameter and the baseline parameter value before treatment was analyzed by Spearman´s correlation. The slopes and intercepts under Chitosan treatment were compared with values under placebo treatment using linear regression analysis.

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3. Results

3.1. Comparison of the baseline data of the Chitosan treated group and the placebo treated group

For all parameters studied, plasma concentrations and marker/cholesterol ratios in the Chitosan group before treatment were not statistically different from these in the placebo group before treatment (Table 1).

Table 1. Comparison of baseline data of the Chitosan group and placebo group; data are expressed as *P*- values obtained with the Mann-Whitney test.

	Placebo	**Chitosan**	***P*-value Chitosan vs Placebo**
Weight (kg)	93.3 ± 13.8	95.7 ± 11.6	0.1594
BMI (kg/m²)	31.6 ± 2.3	31.8 ± 2.3	0.6864
Plasma total cholesterol (mg/dl)	216 ± 49.7	209 ± 42.5	0.3430
Plasma LDL-cholesterol (mg/dl)	131 ± 39.8	129 ± 35.3	0.7213
Plasma HDL-cholesterol (mg/dl)	54.7 ± 16.8	53.5 ± 13.9	0.7525
R_campesterol (µg/mg)	21.6 ± 5.73	22.3 ± 5.75	0.3515
R_sitosterol (µg/mg)	1.24 ± 0.56	1.25 ± 0.47	0.8552
R_cholestanol (µg/mg)	1.08 ± 0.29	1.14 ± 0.34	0.2629
R-lathosterol (µg/mg)	1.66 ± 0.66	1.62 ± 0.53	0.7758
R-lanosterol (ng/mg)	141 ± 40.1	132 ± 30.4	0.3360
R_desmosterol (µg/mg)	0.76 ± 0.35	0.70 ± 0.19	0.7090
R_7αOH-cholesterol (µg/mg)	21.5 ± 46	22.2 ± 13.2	0.8466
R_27OH-cholesterol (µg/mg)	74.7 ± 16.7	78.9 ± 19.8	0.2367

3.2. Weight and BMI

The reduction in weight and BMI (Table 2a and 2b) was statistically significant with placebo ($P<0.0001$) or Chitosan treatment ($P<0.0001$). The changes obtained in the placebo group and Chitosan group were not statistically different from each other. During placebo treatment 90.9% and during Chitosan treatment 88.5% of the subjects underwent weight reduction. Regarding BMI, 81.8% and 80.3% showed reduction in this respect with placebo and Chitosan treatment, respectively. The degree of weight reduction under Chitosan treatment was highly and positively dependent (Figure 1) on the baseline value before treatment (Spearman R= 0.3349, $P=0.0083$). This was not the case in placebo-treated subjects. BMI reduction was not associated with the baseline value neither under Chitosan treatment nor under placebo treatment.

Table 2a. Comparison of changes in body weight induced by Chitosan and placebo treatments. .

Changes in	**body weight**		
	placebo$^{\Delta}$	chitosan$^{\Delta}$	*P*-value* chitosan vs placebo
All subjects (kg)	-3.35 ± 2.51***	-3.51 ± 3.64***	0.7234
Decrease (kg)	-3.73 ± 2.30	-4.13 ± 3.39	0.9042
Increase (kg)	0.48 ± 0.27	1.16 ± 1.58	0.4430
% Subjects decrease	90.9	88.5	0.7660
% Subjects increase	9.1	11.5	
Ratio decrease/increase	10.0	7.71	

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Change vs baseline			
Spearman R	-0.0918	0.3349	
P-value	0.5051	0.0083	
Difference slope from zero	0.1796	0.0157	0.0060

[Δ]Wilcoxon *P*-value expressing the significance of the change compared to zero. *** *P*<0.001 *Data are expressed as *P*- values by using Mann-Whitney test.

Table 2b. Comparison of changes in BMI induced by Chitosan and placebo treatments. .

Changes in	**BMI**		
	placebo[Δ]	chitosan[Δ]	*P*-value[#] chitosan vs placebo
All subjects (kg/m²)	-1.08 ± 0.89***	-0.95 ± 1.73***	0.539
Decrease (kg/m²)	-1.37 ± 0.70	-1.44 ± 1.19	0.6438
Increase (kg/m²)	0.21 ± 0.43	1.16 ± 2.06	0.097
% Subjects decrease	81.8	80.3	1.000
% Subjects increase	18.2	19.7	
Ratio decrease/increase	4.50	4.08	
Change vs baseline			
Spearman R	-0.2397	-0.1505	
P-value	0.0780	0.2470	
Difference slope from zero	0.0781	0.1703	0.7124

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. *** *P*<0.001 [#]Data are expressed as *P*- values by using Mann-Whitney test.

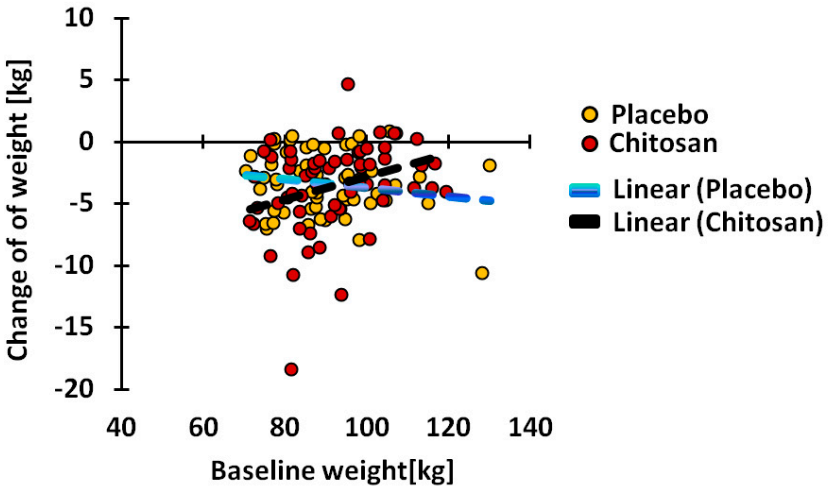

Figure 1. Dependence of the change of weight on baseline weight value under Chitosan and placebo treatment. Under Chitosan treatment the change is significantly (*P*=0.0083) and positively associated with the baseline value.

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3.3. Plasma total cholesterol

Plasma total cholesterol (Table 3) did not decrease under placebo treatment (-5.13 ± 24.79 mg/dl, NS) but decreased significantly under Chitosan treatment (-12.51 ± 28.22 mg/dl, *P*=0.0007). Both changes were not significantly different from each other. The number of subjects undergoing a decrease was also not different: 63.0% under placebo treatment and 67.2% during Chitosan treatment. By both treatments the reduction was significantly and negatively associated with the baseline value. The slopes and intercepts were not significantly different.

Table 3. Comparison of changes in plasma total cholesterol induced by Chitosan and placebo treatments.

Change in	Plasma total cholesterol		
	placebo$^{\Delta}$	chitosan$^{\Delta}$	*P*-value$^{\#}$ chitosan vs placebo
all subjects (mg/dl)	-5.13 ± 24.79	-12.51 ± 28.22***	0.3336
decrease (mg/dl)	-19.44 ± 14.26	-25.34 ±24.27	0.5545
increase (mg/dl)	19.20 ± 19.27	13.80 ± 13.85	0.2077
% subjects decrease	63.0	67.2	0.6968
% subjects increase	37.0	32.8	
Ratio decrease/increase	1.70	2.05	
change vs baseline	-0.4792	-0.3587	
Spearman R	0.0002	0.0045	
P-value	0.0002	0.0066	0.9399
Difference slope from zero			0.0553

$^{\Delta}$ Wilcoxon P-value expressing the significance of the change compared to zero. *** *P*<0.001 $^{\#}$Data are expressed as *P*- values by using Mann-Whitney test.

3.4. Plasma LDL-cholesterol

Plasma LDL-cholesterol (Table 4) decreased significantly under Chitosan treatment (-8.67 ±18.18 mg/dl, *P*=0.0003), but not under Placebo treatment (-1.00 ± 24.22 mg/dl, *P*=0.5613). The reduction induced by Chitosan was significantly larger than the reduction induced by placebo (*P*=0.0252). During placebo treatment the LDL-cholesterol concentration decreased in 48.2% of the subjects, whereas during Chitosan treatment the value was reduced in 73.8% (*P*=0.0076). Interestingly the mean reduction in the subjects undergoing a reduction and mean increase in those showing an increase were both significantly higher in the Chitosan-treated group (both *P*<0.0001). Only in Chitosan-treated subjects the change was highly significantly (*P*=0.0014) and negatively associated with baseline value (Figure 2).

Table 4. Comparison of changes in plasma LDL cholesterol induced by Chitosan and placebo treatments.

Change in	Plasma LDL cholesterol		
	placebo$^{\Delta}$	chitosan$^{\Delta}$	*P*-value$^{\#}$ chitosan vs placebo
all subjects (mg/dl)	-1.00 ± 24.22	-8.67 ± 18.18***	0.0252
decrease (mg/dl)	-2.57 ± 2.36	-16.13 ± 14.01	<0.0001
increase (mg/dl)	1.58 ± 1.29	12.31 ± 10.50	<0.0001
% subjects decrease	48.2	73.8	0.0076
% subjects increase	51.8	26.2	

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Ratio decrease/increase change vs baseline	0.93	2.82	
Spearman R	0.03768	-0.3995	
P-value	0.7868	0.0014	
Difference slope from zero	0.7797	0.0024	0.0019

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. *** $P<0.001$ [#]Data are expressed as *P*- values by using Mann-Whitney test.

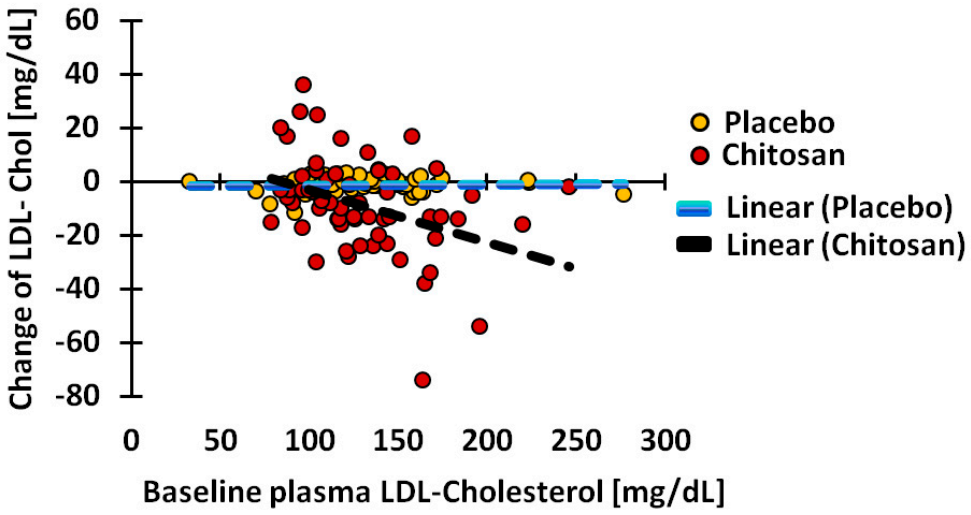

Figure 2. Dependence of change of plasma LDL-cholesterol concentration on baseline value under Chitosan and placebo treatment. Under Chitosan treatment the change is significantly (P=0.0014) and negatively associated with the baseline value.

3.5. Plasma HDL-cholesterol

Plasma HDL-cholesterol did not significantly change under both placebo and Chitosan treatment (Table 5). The observed changes in both treatment groups did not differ from each other. Also the number of subjects experiencing a decrease or increase was similar.

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Table 5. Comparison of changes in plasma HDL cholesterol induced by Chitosan and placebo treatments.

Change	Plasma HDL cholesterol		
	placebo[Δ]	chitosan[Δ]	*P*-value[#] chitosan vs placebo
all subjects (mg/dl)	-1.06 ± 6.81	-1.15 ± 7.65	0.8701
decrease (mg/dl)	-5.97 ± 3.49	-6.88 ± 4.96	0.6973
increase (mg/dl)	5.08 ± 4.56	5.17 ± 4.38	0.9142
% subjects decrease	55.6	52.5	0.8516
% subjects increase	44.4	47.5	
Ratio decrease/increase	1.25	1.10	
change vs baseline			
Spearman R	-0.5054	-0.1491	
P-value	<0.0001	0.2513	
Difference slope from zero	0.0004	0.181	0.9648

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. [#]Data are expressed as *P*-values by using Mann-Whitney test.

3.6. Cholesterol absorption markers

Due to significant decreases of plasma total cholesterol under both placebo and Chitosan treatment, only the marker concentrations corrected for the cholesterol concentration R_campesterol, R_sitosterol, and R_cholestanol, were considered. The changes of the cholesterol absorption marker sterols (Tables 6a-c) during both treatments were not significant from zero except for reduction of R_cholestanol (Table 6c) under placebo treatment. Also changes found under Chitosan treatment were not different from those under placebo treatment. For all three marker compounds in both groups the changes were significantly and negatively associated with baseline values. However, the slopes and intercepts were not different between treatment groups.

Table 6a. Comparison of changes in plasma R_campesterol induced by Chitosan and placebo treatments.

Changes in	R_campesterol		
	placebo[Δ]	chitosan[Δ]	*P*-value[#] chitosan vs placebo
all subjects (ug/mg)	-0.04 ± 0.51	-0.12 ± 0.50	0.3333
decrease (ug/mg)	-0.40 ± 0.42	-0.48 ± 0.35	0.2211
increase (ug/mg)	0.34 ± 0.26	0.32 ± 0.24	0.9036
% subjects decrease	50.9	55.7	0.7097
% subjects increase	49.1	44.3	
Ratio decrease/increase	1.04	1.26	
change vs baseline			
Spearman R	-0.4901	-0.4040	
P-value	0.0001	0.0012	
Difference slope from zero	< 0.0001	0.0084	0.6112

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. [#]Data are expressed as *P*-values by using Mann-Whitney test.

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Table 6b. Comparison of changes in plasma R_sitosterol induced by Chitosan and placebo treatments.

Changes in	R_sitosterol		
	placebo [Δ]	chitosan [Δ]	*P*-value [#] chitosan vs placebo
all subjects (ug/mg)	-0.06 ± 0.27	-0.06 ± 0.36	0.9471
decrease (ug/mg)	-0.24 ± 0.25	-0.31 ± 0.25	0.1668
increase (ug/mg)	0.13 ± 0.09	0.25 ± 0.20	0.0580
% subjects decrease	52.7	54.1	1.0000
% subjects increase	47.3	15.9	
Ratio decrease/increase	1.12	1.18	
change vs baseline			
Spearman R	-0.4224	-0.3557	
P-value	0.0013	0.0049	
Difference slope from zero	< 0.0001	0.0027	0.9860

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. [#]Data are expressed as *P*-values by using Mann-Whitney test.

Table 6c. Comparison of changes in plasma R_cholestanol induced by Chitosan and placebo treatments.

Changes in	R_cholestanol		
	placebo [Δ]	chitosan [Δ]	*P*-value [#] chitosan vs placebo
all subjects (ug/mg)	0.07 ± 0.26*	0.02 ± 0.24	0.255
decrease (ug/mg)	-0.20 ± 0.15	-0.18 ± 0.14	0.8717
increase (ug/mg)	0.18 ± 0.20	0.18 ± 0.18	0.9165
% subjects decrease	30.9	42.6	0.2485
% subjects increase	69.1	57.4	
Ratio decrease/increase	0.45	0.74	
change vs baseline			
Spearman R	-0.4346	-0.5283	
P-value	0.0009	*P*<0.0001	
Difference slope from zero	0.0009	< 0.0001	0.9082

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. * *P*<0.05 [#]Data are expressed as *P*- values by using Mann-Whitney test.

3.7. Cholesterol synthesis markers

Due to the significant decreases of plasma total cholesterol under both placebo and Chitosan treatment, only the marker concentrations corrected for the cholesterol concentration R_lathosterol, R_lanosterol, and R_desmosterol were considered (table 7a-c). R_lathosterol was significantly decreased under Chitosan treatment only (*P*=0.0334). The difference between Chitosan treatment and placebo treatment reached only a trend (*P*=0.0759). R_lathosterol decreased in 49.1% of the subjects under placebo treatment and in 59.0% under Chitosan treatment (NS). R_lanosterol and R-desmosterol were not significantly changed and both treatment changes were not different. During both treatments a negative association between change and baseline value were observed for all three markers, but the slopes were not different. In contrast to R_lathosterol and R_demosterol, R_lanosterol underwent increases in both placebo and Chitosan groups. In subjects that actually experienced a decrease of R_lanosterol, the decrease was significantly less under Chitosan treatment (*P*=0.0324). But relatively more Chitosan-treated

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subjects experienced a decrease: 55.7% vs 38.2% in placebo-treated subjects. This difference reached a trend (*P*=0.0654).

Table 7a. Comparison of changes in plasma total R_lathosterol induced by Chitosan and placebo treatments.

Changes in	R_lathosterol		
	placebo [Δ]	chitosan [Δ]	*P*-value[#] chitosan vs placebo
all subjects (ug/mg)	0.01 ± 0.40	-0.11 ± 0.37*	0.0759
decrease (ug/mg)	-0.28 ± 0.26	-0.34 ± 0.28	0.2139
increase (ug/mg)	0.29 ± 0.28	0.18 ± 0.18	0.2505
% subjects decrease	49.1	59.0	0.3513
% subjects increase	51.9	41.0	
Ratio decrease/increase	0.95	1.44	
change vs baseline Spearman R	-0.2610	-0.5023	
P-value	0.0542	<0.0001	
Difference slope from zero	0.0005	< 0.0001	0.5432

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. * *P*<0.05 [#]Data are expressed as *P*- values by using Mann-Whitney test.

Table 7b. Comparison of changes in plasma total R_lanosterol induced by Chitosan and placebo treatments.

Changes in	R_lanosterol		
	placebo[Δ]	chitosan[Δ]	*P*-value[#] chitosan vs placebo
all subjects (ng/mg)	0.72 ± 26.59	2.30 ± 29.99	0.4588
decrease (ng/mg)	-29.12 ± 24.81	-16.58 ± 17.72	0.0324
increase (ng/mg)	19.14 ± 11.99	26.07 ± 24.98	0.6062
% subjects decrease	38.2	55.7	0.0654
% subjects increase	61.8	44.3	
Ratio decrease/increase	0.62	1.26	
change vs baseline Spearman R	-0.1861	-0.4054	
P-value	0.1738	0.0012	
Difference slope from zero	0.0004	0.0070	0.9836

[Δ] Wilcoxon P-value expressing the significance of the change compared to zero. [#]Data are expressed as *P*-values by using Mann-Whitney test.

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Table 7c. Comparison of changes in plasma total R_desmosterol induced by Chitosan and placebo treatments.

Changes in	R_desmosterol		
	placebo$^{\Delta}$	chitosan$^{\Delta}$	*P*-value$^{\#}$ chitosan vs placebo
all subjects (ug/mg)	-0.04 ± 0.25	-0.04 ± 0.16	0.3616
decrease (ug/mg)	-0.17 ± 0.29	-0.13 ± 0.10	0.5228
increase (ug/mg)	0.10 ± 0.09	0.12 ± 0.09	0.362
% subjects decrease	52.7	62.3	0.3485
% subjects increase	47.3	37.7	
Ratio decrease/increase	1.12	1.65	
change vs baseline			
Spearman R	-0.2577	-0.5399	
P-value	0.0575	*P*<0.0001	
Difference slope from zero	< 0.0001	0.0002	0.8998

$^{\Delta}$ Wilcoxon P-value expressing the significance of the change compared to zero. $^{\#}$Data are expressed as *P*-values by using Mann-Whitney test.

3.8. Bile acid synthesis markers

R_7α-hydroxycholesterol was significantly reduced only under Chitosan treatment (*P*=0.0196) (Table 8a). The changes induced by both treatments were not significantly different from each other. During placebo treatment R_7α-hydroxycholesterol was reduced in 67.3% of subjects, while during Chitosan treatment in 60.7% of the subjects (NS). No significant changes were seen in R_27-hydroxycholesterol in both groups (Table 8b). The changes induced by the two treatments were not significantly different from each other. The relationships between changes and baseline values were not different between placebo and Chitosan treatment, neither for R_7α-hydroxycholesterol, nor for R_27-hydroxycholesterol.

Table 8a. Comparison of changes in R_7αOH-cholesterol induced by Chitosan and placebo treatments.

Changes in	R_7αOH-cholesterol		
	placebo$^{\Delta}$	chitosan$^{\Delta}$	*P*-value$^{\#}$ chitosan vs placebo
all subjects (ug/mg)	0.29 ± 122.85	-28.64 ± 102.15*	0.5541
decrease (ug/mg)	-53.19 ± 42.48	-85.53 ± 72.57	0.1049
increase (ug/mg)	110.23 ± 131.61	59.06 ± 71.18	0.0862
% subjects decrease	67.3	60.7	0.5622
% subjects increase	32.7	39.3	
Ratio decrease/increase	2.06	1.54	
change vs baseline			
Spearman R	-0.3006	-0.6175	
P-value	0.0257	<0.0001	
Difference slope from zero	0.1934	< 0.0001	0.2153

$^{\Delta}$ Wilcoxon P-value expressing the significance of the change compared to zero. * *P*<0.05 $^{\#}$Data are expressed as *P*- values by using Mann-Whitney test.

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Table 8b. Comparison of changes in R_7αOH-cholesterol induced by Chitosan and placebo treatments.

Change	R_27OH-cholesterol		
	placebo$^{\Delta}$	chitosan$^{\Delta}$	*P*-Value$^{\#}$ chitosan vs placebo
all subjects (ug/mg)	-7.32 ± 75.67	-12.71 ± 83.28	0.7743
decrease (ug/mg)	-69.63 ± 53.57	-64.32 ± 68.05	0.3108
increase (ug/mg)	48.55 ± 40.53	56.77 ± 39.96	0.5055
% subjects decrease	47.27	57.37	0.3521
% subjects increase	52.73	42.63	
Ratio decrease/increase	0.90	1.35	
change vs baseline			
Spearman R	-0.2773	-0.3529	
P-value	0.0404	0.0053	
Difference slope from zero	0.0632	0.0012	0.4827

$^{\Delta}$ Wilcoxo*n* P-value expressing the significance of the change compared to zero. $^{\#}$Data are expressed as *P*-values by using Mann-Whitney test.

4. Discussion

This paper describes changes of plasma sterols in 61 highly overweight and obese subjects after Chitosan treatment. The study is placebo controlled with a placebo group consistingof 55 subjects. The absolute baseline and cholesterol-corrected sterol concentrations in both treatment groups at baseline were not different. To identify whether a parameter was selectively affected by Chitosan treatment, following criteria were investigated.

1. The mean change of the parameter value under Chitosan treatment was compared to mean change induced by placebo treatment.
2. For both treatments the parameter were compared to respective baseline values.
3. The percentage of subjects presenting a positive change (increase) or negative change (decrease) under Chitosan treatment was compared to the percent of subjects under placebo treatment.
4. Associations between change of parameter and baseline parameter were determined and compared under placebo and Chitosan treatment. Different associations are indicative of different mechanisms of action.

4.1. Body weight

Both weight and BMI decreased highly significantly under both treatments, but the reductions were not more pronounced under Chitosan treatment compared to placebo . The percentage of subjects undergoing a decrease was also similar in both treatment groups. These data confirm human data obtained in previous studies [5,12,24]. Interestingly, under Chitosan treatment but not under placebo treatment, the weight change was highly significantly and positively associated with baseline weight values indicating that the highest reduction is obtained at the lowest weight. It may point out that the selection of strongly overweight and obese subjects was not the best choice to show a weight reduction effect of Chitosan treatment. Furthermore, Chitosan treatment may be most efficient as a weight gain prevention therapy in subjects of overweight. The correlation data also suggest that the weight reduction due to placebo and Chitosan treatments are established with different mechanisms.

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4.2. Plasma cholesterol concentrations

Plasma total cholesterol decreased significantly under Chitosan treatment only. This decrease was not significantly different from the decrease obtained with placebo treatment, but much more pronounced (P=0.0007) compared to Placebo treatment (P=0.0553). Therefore, a partial Chitosan dependent effect could be assumed. However, the percentage of subjects undergoing a total cholesterol reduction was only slightly higher in the Chitosan treated group. The correlation data did not indicate a trend to assume that different mechanisms explain the concentration reduction under placebo and Chitosan treatments. LDL-cholesterol decreased stronger under Chitosan treatment than under placebo treatment (P=0.0252). Compared to the baseline situation, only Chitosan induced a significant decrease (P<0.0003) in this parameter. Also the percentage of subjects undergoing a decrease of LDL-cholesterol was significantly higher in the Chitosan treated subjects (73,8%) than in placebo treated subjects (48.2%). Thus, according to all three criteria a clear Chitosan induced 5.6% reduction of LDL-cholesterol is achieved vs. 0,9% under Placebo treatment. Assuming that the reduction of LDL-cholesterol is due to trapping of dietary cholesterol in the stomach and intestine, it is of interest to relate this number with the 13% LDL-cholesterol reduction in vegan subjects who ingest 90% less cholesterol with the diet compared to omnivores [22]. Lacto vegetarians had a 44% lower cholesterol intake but not a lower plasma LDL-cholesterol values [25]. From these numbers a trapping efficiency of 60 to 70% of the dietary cholesterol is predicted under Chitosan treatment.

4.3. Surrogate markers of cholesterol absorption

The major plasma markers of cholesterol absorption are cholestanol and plant sterols campesterol and sitosterol. Plant sterols are known to undergo similar changes in absorption as cholesterol. Only the campesterol concentration showed selectively lowered values under Chitosan treatment. However, plasma cholesterol decreased significantly under both treatments. After correcting the plant sterol concentrations for the cholesterol concentrations, no significant differences remained. Also no differences were found between changes due to Chitosan and placebo. In a recent publication [26] we could show that the plant sterol / cholesterol ratio is a good and sensitive reflection of the fractional cholesterol absorption rate measured with stable isotope tracers. Comparing vegan subjects with omnivores [25] did lead to a slightly but significantly lower fractional cholesterol absorption rate in vegans (42% vs 50%) and a largely lowered (90%) dietary cholesterol intake, but not to a change in R_campesterol, R_sitosterol or R_cholestanol. This may be caused by a potentially high intake of plant sterols in vegans. Ezetimibe treatment leads to more than a 50% reduction of the fractional cholesterol absorption and a significantly reduced R_campesterol and R_sitosterol but not R_cholestanol [26]. Importantly, ezetimibe also affects the absorption of biliary cholesterol which amounts 2-3 times more than dietary cholesterol whereas a vegan diet and possibly also Chitosan treatment affects dietary cholesterol only. In view of the available data, the results suggest that unlike ezetimibe Chitosan treatment does not significantly affect cholesterol absorption.

4.4. Surrogate markers of cholesterol synthesis

Three markers for cholesterol synthesis were measured in plasma: lathosterol, desmosterol and lanosterol. Changes observed for desmosterol and lanosterol disappeared after correction for the cholesterol concentration. The ratio R_lathosterol decreased under Chitosan treatment but not under placebo treatment. The decrease observed for Chitosan treatment was not significantly different from the decrease observed for placebo treatment. However, with a P-value of 0.0759, a trend towards reduced cholesterol synthesis was indicated. Of the Chitosan treated subjects 59% had a lower R_lathosterol, which was not significantly higher than the 49% in the placebo group. The results can be interpreted as an

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indication of a small 3% decrease in cholesterol synthesis under Chitosan treatment. At least the data do not indicate an increased synthesis as was hypothesized. The results may be compared with data in lacto vegetarians and vegans as recently described and measured with stable isotope techniques [25]. Lacto vegetarians had a 22% higher cholesterol synthesis than omnivores without a reduction in LDL-cholesterol, vegans a 35% higher synthesis and a 13% lower LDL-cholesterol. However, these diet-induced differences in cholesterol synthesis did not lead to modifications in R_desmosterol and R_lathosterol ratios. As described before [26,27], the surrogate markers R_lathosterol and R_desmosterol for cholesterol synthesis are not sensitive enough to detect relatively small changes in synthesis during cholesterol lowering therapy and reflect primarily hepatic synthesis. Lowering daily intake of cholesterol or fractional absorption of cholesterol may lead to a preferentially enhanced synthesis in intestinal cells. Despite no significant changes, R_lanosterol showed a trend to decrease in more subjects under Chitosan treatment (55,7% vs 38,2% under placebo treatment, P=0.0654). The data suggest that a small decrease of cholesterol synthesis may be initiated by Chitosan. They do not indicate an increased cholesterol synthesis as was hypothesized.

4.5. Surrogate markers of bile acid synthesis

7α- and 27-hydroxycholesterol are markers for bile acid synthesis and 7α-hydroxycholesterol represents the major route of bile acid synthesis. The ratio R_7α-hydroxycholesterol was significantly reduced under Chitosan treatment only (P=0.0196) but not significantly larger than under placebo treatment. R_27-hydroxycholesterol did not change significantly during both treatments and both changes were not different. The percentages of subjects undergoing a R_7α- or R_27-hydroxycholesterol reduction or increase were not different under both treatments. The associations between change and baseline value were significant for 7αOH cholesterol and 27OH-cholesterol under both placebo and Chitosan treatment. However, the slopes and intercepts were not different under both treatments. Therefore, our data do not support an independent Chitosan effect on bile acid synthesis.

4.6. Placebo effects vs Chitosan effects

R_cholestanol decreased significantly under placebo treatment. Other sterols (plasma total cholesterol, LDL-cholesterol, R_lathosterol and R_7α-hydroxycholesterol), were significantly reduced under Chitosan treatment only which suggests an independent Chitosan effect. However, the changes during both treatments were not significantly different, except for LDL-cholesterol. Therefore, the reduction of plasma LDL-cholesterol under Chitosan treatment was the only proven independent effect. Body weight and BMI were also significantly reduced during placebo treatment. These reductions can be explained by the fact that the participants have been advised how to improve the quality of their nutrition and energy expenditure. However, they could eat as usual and more importantly the dietary compliance was not monitored. Interestingly, the reductions in weight and BMI did not differ between placebo and Chitosan treated subjects. Significant changes compared to baseline were observed in both groups (P<0.0001). If the nutritional information is the cause of weight reduction, the mechanism of action should be the same for both treatment groups. However, the significant positive association between the change in body weight and baseline value under Chitosan treatment only suggests a selective mechanism of action. A question is whether the placebo tablet composition may have led to effects. The 55 subjects receiving placebo ingested 8 times 122,50 mg or 980 mg microcristalline cellulose and 8 times 372,50 mg or 2980 mg calcium hydrogenphosphate per day. Cellulose is a solid non- soluble fiber with a low but potential capacity to bind sterols. Cellulose, non-digestible for humans, is fuel for the colonic microbiota and a product of their fermentation are the short-chain fatty acids influencing health, blood lipid profiles and reducing body weight [28]. Calcium hydrogenphosphate is a proton donor applied in baking powder. The potential effect of a daily dosage of 3 grams cannot be simply predicted.

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The dose of Chitosan applied in this study was 4 times the dose used in another study with the same Chitosan product but in combination with a high protein formula replacement of a meal once a day [12]. The placebo group also consumed the meal replacement. The same placebo tablet was used as in the present study, but at a four times lower dose. In this study, plasma total cholesterol and LDL-cholesterol significantly decreased in the Chitosan treated group only. In both cases the changes introduced by Chitosan were significantly larger than by placebo.

The results of the present study do not give an explanation for the reduced plasma LDL-cholesterol concentration under Chitosan treatment. The hypotheses that Chitosan treatment creates a reduced absorption of dietary cholesterol partly compensated by an increased cholesterol synthesis rate could not be proven applying the surrogate marker technology. The question remains whether the applied experimental protocol and the measurement of surrogate markers for cholesterol absorption and synthesis have been sufficiently appropriate to test the hypotheses. From previous studies it could have been predicted that the reductions of plasma total cholesterol and LDL-cholesterol would be small, in the order of a few percent. The choice of a placebo controlled study implies the difficulty to differentiate adequately between a placebo effect and a selective Chitosan effect. The difficulty becomes larger when the differences are small. There is still discussion on the validity of surrogate markers for cholesterol absorption and synthesis under cholesterol lowering therapies [26,27]. In particular, the sensitivity of cholesterol synthesis markers may be considered too low to detect small changes. Furthermore, these markers are considered to represent hepatic cholesterol synthesis. A computer-randomized, double-blind, placebo-controlled, 4-period, balanced, crossover study should be initiated combined with appropriate measurement of daily cholesterol intake and fecal excretion of neutral and acidic sterols as well as plant sterols. A continuous stable isotope feeding method to accurately determine the fractional cholesterol absorption and cholesterol balance procedure to measure cholesterol synthesis should be applied. This approach will give maximal information on independent effects of Chitosan, in particular when participants are fed at the metabolic ward with a strictly controlled diet. Using the same approach various dependencies such as the used Chitosan product (composition, dose, % deacetylation, viscosity index), body weight of studied subjects and experimental conditions (caloric restriction, altered diet composition, altered energy expenditure, normo vs hypercholesterolemic state) should be investigated. Based on these answers the optimal formula and the optimal target patient group for treatment can be assigned.

Interesting alternative modes of action of Chitosan have been recently presented [2] that may affect cholesterol metabolism independently of absorption and synthesis. Some of the mechanisms may be based on general characteristics of fibers: delay of gastric emptying, increased satiety, reduction of appetite, modulation of incretin secretion. Apparently Chitosan treatment can lead to delayed gastric emptying through the highly viscous gel formation and to increased satiety. The latter may lead to decreased food intake as was shown in mice [17]. Most human studies on Chitosan effects deal with the effects on body weight, BMI and waist circumference and /or plasma lipid concentrations. Food intake is not generally checked under treatment. Maezaki et al. [19] showed data on a 2 weeks period of treating 8 normal weight subjects with Chitosan incorporated in biscuits which indicated that cholesterol intake decreased from 340 to 276 mg/day but not statistically significant. The question remains what might have happened at a longer Chitosan treatment duration. Chitosan has also been shown to act antibacterially [29,30] and affects colonic fermentation in rats [31] including short chain fatty acid production which may reduce cholesterol synthesis via propionic acid. Chitosan also acts as an antioxidant [32,33].

5. Conclusions

A twelve weeks treatment of highly overweight and obese subjects with 3 gram Chitosan daily did result in significantly lowered plasma LDL-cholesterol but did not alter surrogate plasma markers of

cholesterol absorption, synthesis and catabolism. A small reduction of dietary cholesterol absorption cannot be excluded.

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Conflicts of Interest. The authors declare that they have no conflict of interest. The founding sponsor had no role in the design of the study; in the collection, analyses, or interpretation of the data; in the writing of the manuscript, and in the decision to publish the results.

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