Circulating endotoxin as a potential biomarker and mediator of inflammation: influenced by diabetic therapies

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Circulating endotoxin as a potential biomarker and mediator of inflammation: influenced by diabetic therapies

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Running Title: Endotoxin as an inflammatory mediator in T2DM

Abstract

Chronic low-grade inflammation is a significant factor in the development of obesity associated diabetes. This is supported by recent studies suggesting endotoxin, derived from gut flora, may be key to the development of inflammation by stimulating the secretion of an adverse cytokine profile from adipose tissue. The study investigated the relationship between endotoxin and various metabolic parameters of diabetic patients to determine if anti-diabetic therapies exerted a significant effect on endotoxin levels and adipocytokine profiles of T2DM subjects. Fasting blood samples were collected from consenting Saudi Arabian patients (BMI: 30.2± (SD) 5.6kg/m^2, n=413), consisting of non-diabetics (controls, n=67) and T2DM subjects (n=346). The diabetics were divided into 5 subgroups based on their 1 year treatment regimes: diet-control (n=36), metformin (n=141), rosiglitazone (RSG, n=22), a combined fixed dose of metformin/rosiglitazone (Avandamet n=100) and insulin (n=47). Lipid profiles, fasting plasma glucose, insulin, adiponectin, resistin, TNF-α, leptin, C-reactive protein (CRP) and endotoxin concentrations were determined. Regression analyses revealed significant correlations between endotoxin levels and triglycerides (r^2=0.42; p<0.0001); total cholesterol (r^2=0.10; p<0.001), glucose (r^2=0.076; p<0.0001) and insulin (r^2=0.032; p<0.001). Endotoxin showed a strong inverse correlation with HDL-cholesterol (r^2=0.055; p<0.001). Further, endotoxin levels were elevated in all of the treated diabetic subgroups compared with control, but only the RSG group did not differ significantly from control (Control:4.2±1.7EU/ml, RSG:5.6±2.2EU/ml). Both the Avandamet and RSG treated groups had significantly higher adiponectin levels than all the other groups, with the RSG group expressing the highest levels overall. We conclude that sub-clinical inflammation in T2DM may, in part, be mediated by circulating endotoxin. Furthermore, that whilst the endotoxin and adipocytokine profiles of diabetic patients treated with different therapies were

comparable, the RSG group demonstrated significant differences in both adiponectin and endotoxin levels. We confirm an association between endotoxin and serum insulin and triglycerides and an inverse relationship with HDL. Lower endotoxin and higher adiponectin in the groups treated with RSG may be related and indicate another mechanism for the effects of RSG on insulin sensitivity.

Keywords: Type 2 Diabetes Mellitus, Inflammation, obesity

Introduction

In recent years, obesity, insulin resistance as well as many of the features that comprise the metabolic syndrome are associated with a low-grade, systemic, inflammatory condition (16, 39). In particular, the mediation of this sub-clinical inflammation in the pathogenesis of T2DM is proposed to arise through increasing adiposity (17, 32); with adipose tissue representing a site of an acute phase response (6, 10) through the production of known pro-inflammatory adipocytokines such as leptin, tumor necrosis factor (TNF-α) and interleukin (IL-6), amongst others (1, 34, 35). Specifically, adipocytokines appear to have a duality of function, simultaneously mediating inflammation and insulin resistance through their effects on insulin action. Therefore adipocytokines may function as a consequence of the cross-link proposed between metabolic and inflammatory pathways in adipocytes and immune cells (26, 37, 43).

Current therapies utilized in the treatment of T2DM include metformin and the thiazolidinediones (TZDs). These agents have principally been evaluated on the basis of their beneficial effects on glucose metabolism, due to their insulin enhancing properties. Metformin and rosiglitazone (RSG) are widely accepted first-line anti-diabetic drug therapies, whether taken as a monotherapy or in combination (33), and are considered effective with low incidence of hypoglycemia (38). In addition to their effects on glucose homeostasis, metformin, itself, is known to reduce leptin, with resultant effects on inflammatory status and satiety (24). Furthermore, the TZDs have also previously been shown to have immunomodulatory effects (13); reducing inflammation in both *in-vitro* and *in-vivo* models (8, 30, 36). These findings highlight alternative pathways through which drug therapies are able to counteract the

progressive nature of metabolic disease and their potential dual action on reducing obesity mediated T2DM.

Whilst metformin and the TZDs appear to offer dual functionality in insulin resistance and inflammation, the initial mediator for such an inflammatory insult is less well understood. One source of sub-clinical inflammation in patients with T2DM and coronary heart disease may arise through commensal bacteria derived from the gut, referred to as endotoxin (12). Endotoxin is derived from lipopolysaccharide (LPS) which represents cell wall fragments of gram negative bacteria. Previous studies have demonstrated that subjects with obesity and T2DM have elevated circulating levels of endotoxin compared with non-diabetic controls, which has been implicated in increased inflammatory risk (15). Further support for this concept has arisen in subsequent studies where serum endotoxin levels are significantly higher in *ob/ob* and *db/db* mice compared with their normal weight counterparts (7). Studies suggest elevated endotoxin levels may arise as a result of obesity related hyperinsulinemia; hence low-grade endotoxinemia may be caused by the effect of insulin on intestinal motility and/or intestinal permeability. In support of this theory, murine studies analyzing the gastrointestinal tract (GIT) of *ob/ob* and *db/db* mice identified pathological changes in the GIT cells. The findings indicated that insulin may act directly on the GIT to affect gut permeability and potentially increase endotoxin absorption (7). Elevated circulating levels of endotoxin may then initiate an inflammatory response within adipose tissue, via innate immunity, in conjunction with the liver, as the latter is the primary site of endotoxin clearance under normal physiological circumstances (6, 7). This mechanism may therefore explain the state of sub-clinical inflammation often present in obese and type 2 diabetic subjects (39).

Whilst limited data have been presented on the increase of endotoxin in pathological conditions such as obesity and diabetes, no data, as yet, have evaluated the influence of insulin sensitizers in combination on inflammatory risk posed by circulating endotoxin. Whilst there is a large quantity of data available on obesity and T2DM in relation to the Western world, less information exists regarding populations in the transitional state. In particular, Saudi Arabia has experienced a rapid increase in wealth over a relatively short period of time as a consequence of the financial gains rendered by the oil industry, paralleled with swift industrialization and urbanization (18). As a result, the burden of disease in Saudi Arabia is high. The Obesity Taskforce worldwide projections for 2030 predict that the incidence of diabetes will rise by 32% in Europe, 72% in the USA but will increase by a massive 164% in the Middle East (22, 41) . As such it is clear we need to have a fundamental understanding of their risk of inflammation and how insulin sensitizers, along with other therapies, may reduce such a risk.

Therefore, the aims of this present study were to establish any associations between endotoxin and insulin, glucose, lipid profiles and pro-inflammatory cytokines in a Saudi Arabian cohort. Furthermore, the study aimed to examine the influence of diet, metformin, RSG or a combined-fixed dose of metformin/RSG (Avandamet) as well as insulin on endotoxin levels and its' association with inflammation. Ultimately, this study will evaluate whether alterations in endotoxin, coupled with altered metabolic profile, in type 2 diabetics might further explain the observed improvement in the clinical profiles of patients treated with these therapies. This present study, in combination with our previous findings, may therefore help determine endotoxin as a novel biomarker of T2DM and diabetic risk and hence proffer a new target for the treatment or prevention of diabetes.

Methodology

This single-center, prospective and cross-sectional study was carried out at the Diabetes Center of King Abdul-aziz University Hospital in Riyadh, Kingdom of Saudi Arabia. The study protocol was approved by the institutional review board and was conducted in accordance with the guidelines set by the College of Medicine and Research Center of King Saud University, Riyadh, Kingdom of Saudi Arabia ethics committee. All patients submitted written and informed consent prior to inclusion.

Subjects

The study consisted of a total of 413 out-patients (male: 203; female: 210) age 20-80. Furthermore, 346 subjects were known type 2 diabetics while the remaining 67 were non-diabetics, closely matched for BMI.

Inclusion criteria for the diabetics (prior diagnosis) were determined in the first screening visit and included HBA1c 6-11%, fasting plasma glucose 6.7-13.9mmol/l; BMI 22-40kg/m^2; without co-existing diabetic complications, i.e. diabetic retinopathy, nephropathy, etc. Subjects must have received treatment (diet controlled, metformin alone, RSG alone, a combined fixed dose of metformin and RSG (Avandamet) or insulin alone) for at least one year. Control subjects had normal fasting plasma glucose (<5.6mmol/l); HBA1c levels (4-6%) and were not taking any medications prior to commencement of research. Patients were excluded if they had poorly controlled diabetes with co-existing complications, smoking history, history of coronary heart disease and any unstable medical condition/s that would require immediate attention.

All subjects underwent a complete physical examination, which included an electrocardiogram prior to enrolment. Qualified patients were then stratified into 6 groups on the basis of their hypoglycemic therapies (diet-controlled (n=36), metformin (n=141), RSG (n=22), Avandamet (n=100) and insulin (n=47)), in addition to the non-diabetic control group (n=67).

In vivo Assessment of the Biochemical profile, Adipocytokines and Endotoxin Levels

On the assigned date, fasting blood samples were collected from participating subjects and lipid profiles and fasting plasma glucose determined using routine laboratory methods. Adipocytokine levels were also analyzed. Adiponectin, resistin, TNF-α, leptin and C-reactive protein (CRP) were quantified using various sandwich enzyme-linked immunosorbent assays (ELISAs) (Linco Ltd, USA; RnD Systems, Ltd, UK; Immunodiagnoztik AG, Germany, respectively), whilst insulin was analyzed via a solid phase enzyme amplified sensitivity immunoassay (Medgenix INS-ELISA, Biosource, Belgium). Lastly, endotoxin concentration was measured using a chromogenic kinetic limulus amebocyte assay (LAL assay, BioWhitaker, Walkersville MD), which had been validated, previously (15).

Statistical Analyses

Data were analyzed using the Statistical Package SPSS for Windows version 11.5. Data were expressed as mean ± standard deviation and mean (range) if not normally distributed. Triglycerides, insulin, leptin, adiponectin and resistin were logarithmically transformed to normalize data. Groups were compared using ANOVA with bonferroni adjustments for inter group comparisons. Simple and partial correlation coefficients between the variables were determined and multiple regression analysis was carried out to determine variables of interest.

Results

Metabolic and Clinical Characteristics

Baseline characteristics

Table 1 shows the clinical and metabolic characteristics of the 6 groups analyzed in this study. The mean systolic blood pressures of the groups were similar, with the diet group having the lowest mean systolic blood pressure (111.6 ± 22.9mmHg) and the Avandamet group (127.4 ± 23.6mmHg, $p<0.05$) the highest. The mean diastolic blood pressures of the control (84.1 ± 14.6mmHg) and Avandamet treated groups (84.6 ± 13.0mmHg) were significantly lower than the insulin treated group (96.0 ± 15.5mmHg, $p<0.05$). Waist to hip ratios (WHR) were similar across all groups with the insulin treated group showing the highest WHR (1.4 ± 0.4), that differed significantly from the non-diabetic controls and the Avandamet treated groups (control: 1.1 ± 0.2; Avandamet: 1.1 ± 0.3, $p<0.05$). As would be expected, the non-diabetic control group had significantly lower fasting plasma glucose levels compared to the other 5 groups, which were similar to one another. The groups were also comparable in terms of lipid profile, although triglyceride levels of the metformin and Avandamet treated groups (metformin: 2.1 ± 2.0 mmol/l; Avandamet: 2.2 ± 1.5 mmol/l, $p<0.05$) were significantly higher than the control group (1.4 ± 0.7 mmol/l, table 1).

Associations Between Endotoxin levels and Metabolic Factors in type 2 diabetics

Regression analyses revealed a significant correlation between endotoxin levels and triglycerides ($R^2=0.42$; $p<0.0001$) (figure 1a); an inverse correlation between HDL-cholesterol values ($R^2=0.10$; $p<0.001$) (figure 1b) and a significant correlation between total cholesterol levels ($R^2=0.10$; $p<0.001$) (figure 1c) in the diabetic cohort. Lastly we found a significant association between endotoxin levels and insulin

(r^2=0.032; p<0.001, figure 2a) as well as glucose (r^2=0.076; p <0.001, figure 2b) in the type 2 diabetics. The rest of the analyses were non-contributory.

Circulating Endotoxin Levels in non-diabetic and treated T2DM subjects

Serum endotoxin levels were lowest in the non diabetic control group (4.2±1.7 EU/ml, table 1, figure 3), with the insulin treated cohort demonstrating the highest levels (9.2±4.4 EU/ml, table 1, figure 3). Out of all of the anti-diabetic therapies, only the RSG treated group had circulating endotoxin levels comparable with the non-diabetic controls (RSG: 5.6±2.2 EU/ml, Control: 4.2 ± 1.7 EU/ml, table 1, figure 3). Furthermore, only the RSG treated group showed significantly lower endotoxin levels than the insulin treated group (p<0.05, table 1, figure 3).

Circulating Adipocytokine Levels

With regard to adipocytokines, the leptin levels of the control group (31.0(25-36) ng/ml) were significantly higher compared with the treated type 2 diabetic subgroups (table 1, figure 4). Adiponectin levels in the RSG (16.1(12-20)µg/ml) and Avandamet (14.3(13-16))µg/ml) treated groups were significantly higher than all the other groups, with the RSG group showing almost a two fold increase in adiponectin levels compared with control (4.2 ± 1.7µg/ml), figure 4). The mean resistin levels of the groups were comparable, except for the RSG group which showed the lowest resistin levels overall (11.9(9-14))ng/ml) and differed significantly from the metformin (16.1(15-17)ng/ml) and insulin groups (17.6 (13-22) ng/ml, table 1, figure 4). TNF-α and CRP levels did not vary significantly between any of the groups (data not shown).

Discussion

Recent studies have implicated a role for adipose tissue as a site of systemic inflammation, thus providing a direct link between obesity and the associated state of chronic sub-clinical inflammation (39). One principal source for inflammatory risk may occur via the GIT, as previous studies have determined that endotoxin can activate the innate immune response within adipose tissue (2, 7, 11, 13, 15). Such studies signify a potential role for gut flora related induction of innate immunity and circulating endotoxin in the pathogenesis of obesity induced T2DM.

The findings from this study have highlighted that subjects with T2DM had significantly elevated levels of endotoxin compared with BMI matched non-diabetic controls, affirming previous studies in Caucasian populations whilst increasing the studied subjects numbers four fold (15). Moreover, our findings also demonstrated that insulin treated subjects exhibited the highest circulating endotoxin levels of all the cohorts examined; additionally both insulin and glucose were shown to correlate significantly with endotoxin levels in the diabetic cohort. As such, our current and previous data have highlighted an association between insulin and endotoxin, suggesting a mechanism that hyperinsulinemia may lead to increased absorption of endotoxin through the GIT (7, 40). Therefore, endotoxin may acts as an inflammatory mediator in T2DM (7, 15). Our understanding of the relationship between glucose and endotoxin may appear less clear as previous studies show no correlation between glucose levels and endotoxin (15), with Pederson and colleagues observing only a moderate increase in glucose levels when subjects underwent endotoxin infusion (25). However, severe human sepsis is known to instigate a hypermetabolic stress response which includes hyperglycemia and impaired glucose tolerance (44). Furthermore in T2DM patients, elevated glucose levels interfere with macrophage function, and

suppress the bactericidal capacity of leukocytes (44), whilst murine studies have shown elevated glucose levels cause adverse effects on jejunum motility (9). As such, hyperglycemia may make patients more susceptible to further infection – as observed in T2DM – as well as potentiating endotoxin absorption through inhibition of gut motility. Although patients with septic shock have considerably higher endotoxin levels (10 – 50 fold) than type 2 diabetics (14), subjects with cirrhosis are on par with obese and T2DM subjects (21). Therefore an association between glucose and endotoxin would suggest a role for endotoxin as an inflammatory mediator (29). Our current findings have identified a clear, positive correlation between endotoxin and glucose serum levels (15). The apparent discrepancies between our study and previous findings may have arisen due to the large size of the cohort we examined. Therefore the relationship between endotoxin and glucose should be further investigated in other, sizeable, diabetic populations in order to corroborate our findings.

The strong positive correlation identified between endotoxin and triglyceride levels confirm previous findings (15, 23). Furthermore, our findings regarding HDL cholesterol showed the same inverse relationship with endotoxin as observed in the study by Creely and colleagues (15). Marked reduction in HDL-cholesterol is reported to form part of the lipid-lowering effects of inflammation (23). HDL has been identified as having a protective role against the effects of endotoxin, actively binding, neutralizing and ultimately clearing endotoxin from the body (27). As such, lower HDL levels would make a patient more susceptible to the detrimental effects of the pathogen.

Upon comparing the different treatment regimes, only the RSG group had circulating endotoxin levels comparable with the non-diabetic controls. In addition, only the RSG

treated group showed significantly lower endotoxin levels than the insulin treated group. Taken together, these findings support previous data demonstrating the capacity of RSG to lower circulating endotoxin levels in T2DM subjects (15) which may explain some of the anti-inflammatory properties of the thiazolidinediones (TZDs). RSG and Avandamet treated subjects both resulted in elevated adiponectin levels that, whilst comparable to one another, were significantly higher than all the other treated groups, including control. These data affirm findings by Rosenstock and colleagues and support their suggestion that the observed increase in adiponectin levels is due to RSG, as the TZDs have been shown to consistently increase levels of adiponectin (31).

Assessment of other adipocytokines showed that leptin levels were highest amongst the control groups and did not correlate with endotoxin in all groups. However, to date, the findings regarding leptin are conflicting. Patients with sepsis are known to have elevated levels of leptin (4), yet several studies have failed to demonstrate an increase in leptin with endotoxin infusion (5, 19), whilst studies in mice and hamsters show increased leptin at the serum and mRNA level with endotoxin administration (3, 20). Such differences may have arisen because our study measured the relationship between leptin and endogenous endotoxin serum levels, as opposed to the effects on leptin as a result of exogenous endotoxin administration. Furthermore, within this study, the diabetic drugs may have decreased leptin concentration. Metformin is a leptin-reducing agent and was recently reported to enhance leptin sensitivity (24), as well as RSG (42). In this instance, the drug treatment regimes for T2DM may have influenced the data to alter the determined findings.

In summary, both non-diabetic and diabetic subjects showed significant associations with endotoxin and glucose, insulin, triglycerides and total cholesterol, with an inverse correlation between endotoxin and HDL-cholesterol. As such, endotoxin may mediate various metabolic alterations in people with T2DM in response to an increasing insulin resistant state, which is also consistent with chronic systemic low-grade inflammation. Our data suggest that the sub-clinical inflammation observed in diabetic patients may, in part, be derived from commensal bacteria (28), whilst clinical treatment of T2DM appears to suggest RSG has the most favorable effect on overall endotoxin reduction and adipocytokine profiles in the diabetic patients. Clearly additional studies are required to determine the cause and effect via further examination of the role of endotoxin and the insulin resistant state. However, endotoxin may represent an important biomarker for inflammatory metabolic risk.

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Figure Legends

Table 1. Clinical characteristics for controls and subjects with T2DM on anti-diabetic therapies. Data are presented as mean ± SD unless # as mean (range). § denotes significance compared to diet; ‡ denotes significance compared to insulin; * denotes significance compared to metformin; † denotes significance to Avandia; ** denotes significance compared to Avandamet; Significance set at $p<0.05$. Variables with # were log transformed prior to comparison.

Figure 1. Correlations between log fasting endotoxin (EU/ml) and a) log triglycerides (mmol/l), b) HDL (mmol/l) and c) cholesterol (mmol/l) in the whole diabetic cohort. The lines of best fit are also shown: a) $r^2=0{\cdot}42$, $p<0{\cdot}0001$, b) $r^2=0{\cdot}055$, $p<0{\cdot}001$, c) $r^2=0{\cdot}1$, $p<0{\cdot}001$,).

Figure 2. Correlations between log endotoxin (EU/ml) and a) log fasting insulin (ng/ml) and b) glucose (mmol/l) in the whole diabetic cohort. The lines of best fit are also shown: a) $r^2=0{\cdot}032$, $p<0{\cdot}001$, b) $r^2=0{\cdot}076$, $p<0{\cdot}001$.

Figure 3. Graph to show the mean endotoxin levels (EU/ml) and standard deviation of diabetic patients on various anti-diabetic therapies compared with control. *$p<0{\cdot}05$.

Figure 4. Graph to show the mean levels of adipocytokines: resistin (ng/ml), leptin (ng/ml) and adiponectin (μg/ml) of diabetic patients on various anti-diabetic therapies along with control.

Table 1. Clinical and Metabolic Characteristics of Subjects

Variables	Control	Diet	Insulin	Metformin	Avandia	Avandamet
Age (years)	44.1 ± 9.9‡*†**	48.3 ± 9.1‡	55.6 ± 11.4	53.0 ± 10.5	52.3 ± 9.5	52.5 ± 9.0
BMI (kg/m^2)	30.0 ± 5.2	29.6 ± 5.8	29.0 ± 6.2	32.0 ± 5.8	29.6 ± 5.8	31.0 ± 5.3
Systolic (mmHg)	117.3 ± 20.0	111.6 ± 22.9†	115.7 ± 24.9	123.8 ± 23.5	114.1 ± 28.7	127.4 ± 23.6
Diastolic (mmHg)	84.1 ± 14.6‡	88.2 ± 14.9	96.0 ± 15.5	90.3 ± 14.3	90.9 ± 16.3	84.6 ± 13.0‡
WHR	1.1 ± 0.2 ‡	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.3 ± 0.3	1.1 ± 0.3†‡
Glucose (mmol/l)	5.5 ± 1.5*†‡§**	8.4 ± 1.9	7.1 ± 2.8†	9.6 ± 3.4	8.4 ± 1.9	9.4 ± 3.9
Insulin (ng/ml)	20.1 ± 19.5‡	19.1 ± 10.5‡	29.9 ± 11.2	20.7 ± 15.1‡	21.5 ± 19.2	17.1 ± 20.1 ‡
LDL-C (mmol/l)	3.2 ± 0.9	2.7 ± 0.8	3.0 ± 1.0	2.8 ± 0.8	2.8 ± 0.7	3.0 ± 1.3
HDL-C (mmol/l)	1.2 ± 0.4	1.3 ± 0.7	1.1 ± 0.4	1.1 ± 0.4	1.0 ± 0.4	1.2 ± 0.5
TC (mmol/l)	5.0 ± 1.0	4.7 ± 1.0	4.9 ± 1.1	4.8 ± 1.0	4.4 ± 0.9	5.1 ± 1.2
TG (mmol/l)	1.4 ± 0.7*†	1.6 ± 0.9	1.8 ± 0.8	2.1 ± 2.0	1.6 ± 1.0	2.2 ± 1.5
CRP(μg/ml)#	4.2(3-5)	3.8 (2-5)	6.3 (4-9)	4.6(3-6)	3.7(2-6)	3.6(3-5)
TNF-α (pg/ml)	5.4 ± 2.2	4.7 ± 2.0	5.4 ±3.6	4.2 ± 1.7	5.9 ± 2.3	4.5 ± 1.7
Endotoxin(EU/ml)	4.2 ± 1.7§‡***	7.9 ± 3.6	9.2 ± 4.4	7.5 ± 5.0	5.6 ± 2.2‡	7.4 ± 3.7

Figure 1a

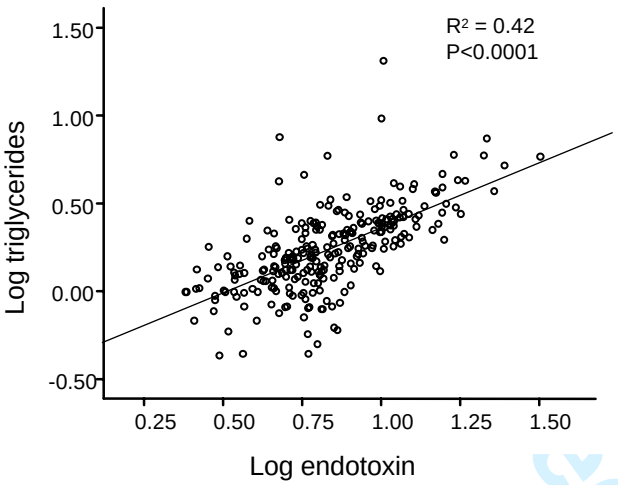

Figure 1b

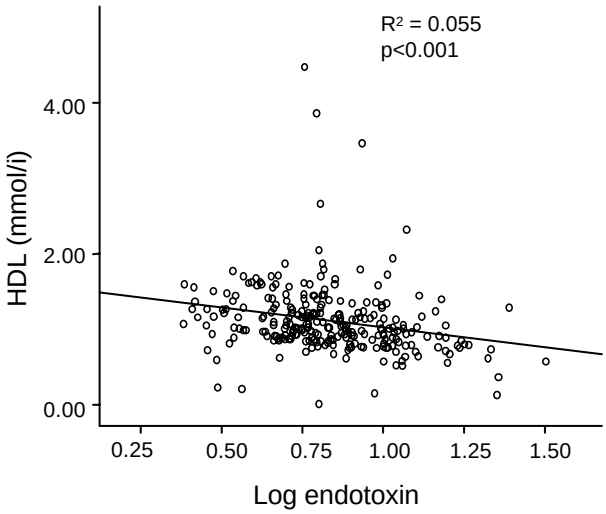

Figure 1c

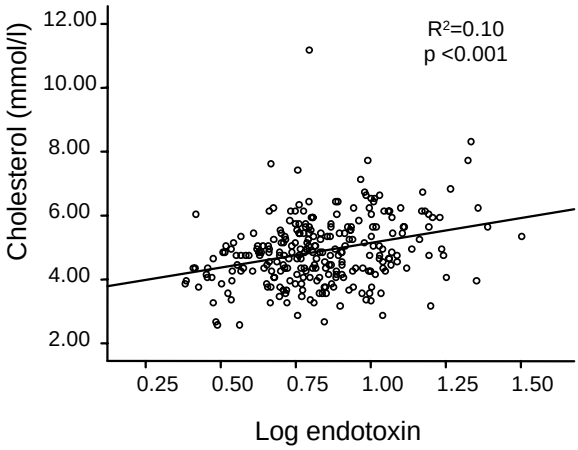

Figure 2a

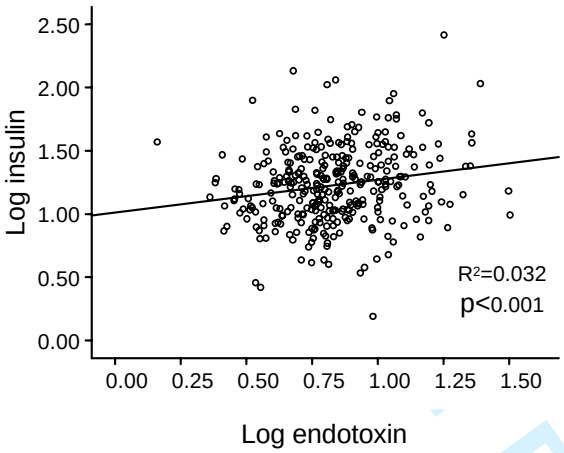

Figure 2b

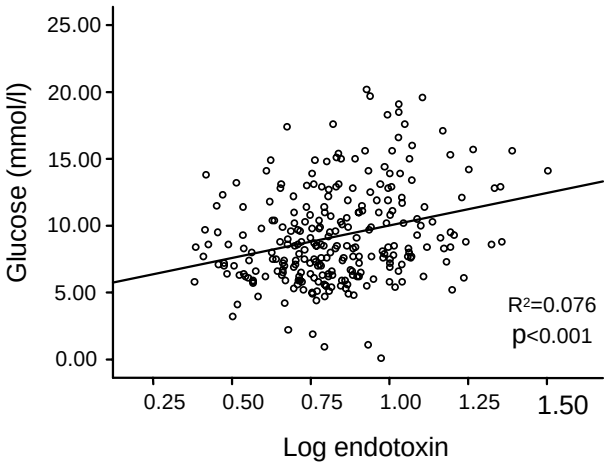

Figure 3.

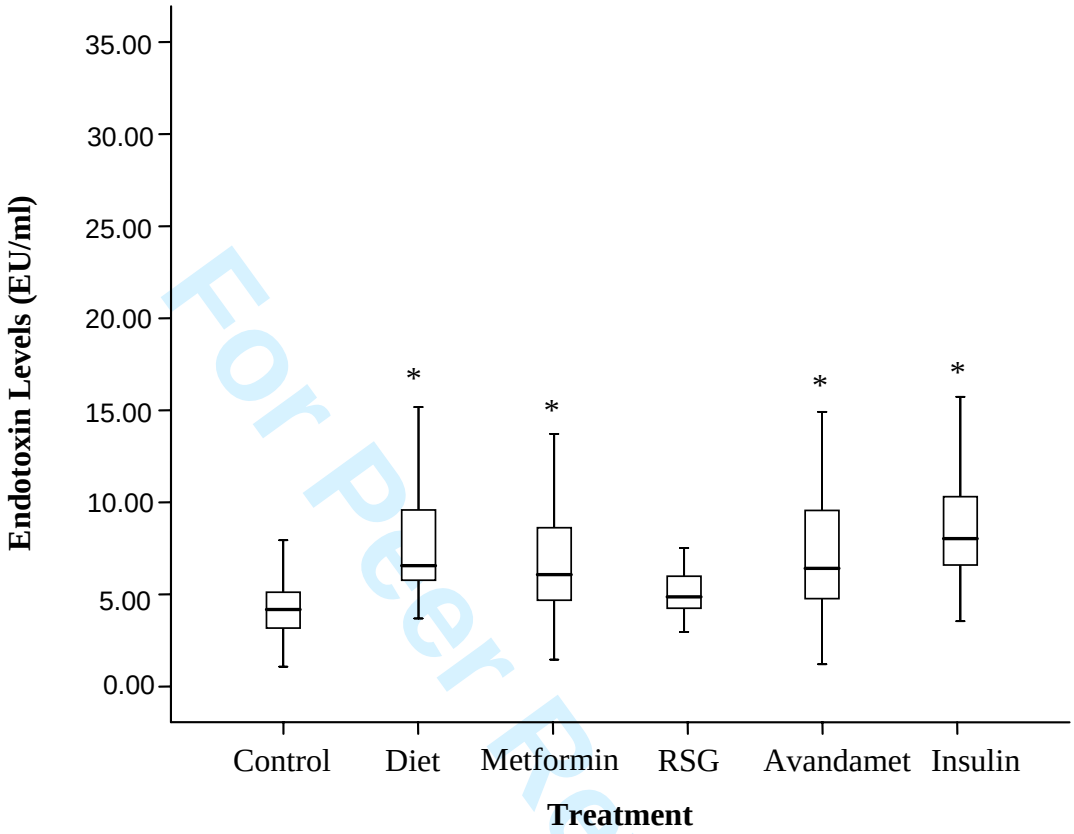

Figure 4

Adipocytokine Profiles of Treatment Groups