# DIAMETRIX DOWN-REGULATES INFLAMMATORY SIGNALING IN OBESE TYPE-2 DIABETES MURINE MODEL IN VIVO

Kenneth R. Hampshire[1], Steven Miles[1], Rick Miles[1], Robert T. Streeper[2], Armando Diaz[2], David Campos[2], Joel Michalek[3], Christopher Louden[3], Wieslaw Furmaga[3], Elzbieta Izbicka[2&]

[1]Syntratech Corporation, Denver, Colorado, USA
[2]CPC LTD, San Antonio, Texas, USA
[3]University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA
[&]corresponding author

## ABSTRACT

**Background:** DiaMetrix, a dietary supplement, lowered blood glucose and improved several surrogate endpoints in a clinical study of diabetic patients. This study compared the activity of DiaMetrix to that of anti-diabetic drugs in an obese diabetic mouse model.

**Methods:** DiaMetrix, metformin, Actos (pioglitazone hydrochloride), and Byetta (exenatide) were administered to BKS.Cg-m+/+Lepr$^{db}$/BomTac female mice. Untreated animals served as a control. Animals (8 per group) were fed either normal diet (ND) or high fat diet (HFD) +/- drugs for 8 weeks. Body mass was measured weekly. We quantified plasma levels of 40 biomarkers (chemokines, cytokines, endocrine, growth factors and metabolites) including insulin, glucose, advanced glycation end product (AGE), cholesterol and triglycerides. Pyruvate kinase activity, citrate, ADP and ATP concentrations and hexokinase II expression were determined in muscle tissue. Organ pathology was assessed microscopically. Mean values of all 45 biomarkers were compared between treatment groups.

**Results:** DiaMetrix's protection against organ damage was comparable to that of Byetta and better than Actos and metformin in the animals on ND. The mean values of most plasma biomarkers were elevated in HFD relative to ND. Biomarker means varied significantly by treatment group and diet. On ND, DiaMetrix decreased levels of eotaxin, MCP-1, MCP-3, M-CSF, and increased IL-4 relative to untreated. DiaMetrix treatment decreased G-CSF, GM-CSF, and TGFβ relative to untreated in HFD animals. Pyruvate kinase and AGE increased, while insulin was decreased in animals treated with DiaMetrix relative to untreated on ND. Treatment group contrasts on biomarkers (MCP-3, IL-17, AGE, and insulin) also varied with diet.

**Conclusions:** DiaMetrix demonstrated superior anti-inflammatory activity relative to the commonly used anti-diabetic drugs against a background of genetic obesity. These results, taken together with the results of histological examination, support the contention that DiaMetrix may be an effective intervention for type-2 diabetes.

**Introduction**

Diabetes mellitus type 2, also known as type 2 diabetes, is an autoimmune-inflammatory disease, which is fueled by pathological expression of the innate immune system in non-immune hypothalamic cells, visceral adipocytes, ß-cells of the pancreas, and vascular endothelium (*1*). Among risk factors, a high fat diet is considered to be detrimental to people with diabetes. Consumption of a fat-rich diet activates a pro-inflammatory response and induces insulin resistance in the hypothalamus (*2*), (*3*). Dietary fat causes high blood sugar levels by preventing sugar from entering muscles and other cells, thus keeping sugar in the bloodstream and elevating it to high levels (*4*), (*5*). In addition, physical inactivity and obesity are two major risk factors for the development of type 2 diabetes (*6*). High blood glucose in the context of insulin resistance and relative insulin deficiency is associated with decreased rates of glycolysis, glycogenesis, lipogenesis, and protein synthesis. Under normal conditions, more than 80% of the energy produced by the body is derived from carbohydrate metabolism. When this metabolism is severely limited, the cell initiates oxidation of fat reserves for energy production and degrades proteins to amino acids which in turn are converted to glucose. If excessive fat metabolism occurs in conjunction with inadequate carbohydrate utilization, there are inadequate amounts of oxaloacetic acid with which to react with acetyl CoA from the fatty acid oxidation. An excess of acetyl CoA leads to a buildup of ketone bodies leading to ketosis and acidosis. Long term complications of the diabetic condition include arteriosclerosis and other cardiovascular problems, changes in the retina and the formation of cataracts, nervous system problems and kidney diseases.

DiaMetrix™ is an herbal dietary supplement composed of vitamin C (ascorbic acid), biotin USP, chromium (chelate), vanadium (chelate), garcinia cambogia extract (50% hydroxycitric acid), gymnema sylvestre extract (25%), cinnamon extract (4:1), bitter melon extract (10:1), betaine HCL, banaba extract (1% corosolic acid), fennugreek, dicalcium phosphate, cellulose, croscarmellose sodium, stearic acid, silicon dioxide, magnesium stearate, and hydroxypropyl methylcellulose. A recent double-blinded clinical study of diabetic patients demonstrated that DiaMetrix lowered blood glucose levels, triglycerides, cholesterol, and blood pressure (Hampshire *et al*, manuscript in preparation). To shed more light on DiaMetrix's mode of action, we compared the activity of DiaMetrix to three commonly used anti-diabetic drugs: metformin, an anti-hyperglycemic agent that has been in use for nearly two decades (*7*) and two more recent drugs; Actos (pioglitazone hydrochloride)(*8*) and Byetta (exenatide) (*9*). These drugs were examined at doses comparable to those used in clinical practice in a murine model using BKS.Cg-*m+/+Lepr*$^{db}$/BomTac (db/db) female mice. These mice are homozygous for a point mutation in the gene for the leptin receptor LEP-R (*10*), a protein that in humans is encoded by the *LEPR* gene. The db/db mouse is a model of obesity, diabetes, and dyslipidemia (*11*) (*12*). LEP-R-deficient db/db mice develop disease similar to human type 2 diabetes mellitus, hypertension, and obesity with disrupted circadian blood pressure rhythm. They are also known to develop hyperglycemia at approximately 6 weeks age and frank immune dysfunction by 20 weeks (*13*).

Using this *in vivo* system, we selected a broad range of endpoints to evaluate mechanisms of action of DiaMetrix. In addition to commonly tested metabolites (glucose, advanced glycation end product, insulin, cholesterol, triglycerides), we included pyruvate kinase, hexokinase II, and citrate as potentially relevant endpoints along with a large panel of cytokines, chemokines, endocrine markers and growth factors.

**Methods**

**Animals:** The study was performed according to the guidelines for the care and use of laboratory animals at CPC LLC, San Antonio, Texas. BKS.Cg-*m+/+Lepr*$^{db}$/BomTac female mice that were 7 to 9 weeks old at shipment were sourced from Taconic Farms (Hudson, NY). The animals were housed until 10 to 12 weeks of age before start of treatment.

**Animal diet and drug treatment:** Animals were randomized into two groups of 40 mice each and fed *ad libitum* with Purina 5001 rodent diet (normal diet; ND) or BioServ High Fat Diet p.n. F1850 (BioServ, Frenchtown, NJ 08825) rodent diet paste (high fat diet; HFD). Each diet group was further divided into five treatment groups (Actos, Byetta, metformin, DiaMetrix, untreated) with 8 animals for each treatment group.

The recommended dosage of DiaMetrix is six capsules per day. Thus, if we refer to the maximum average 75th percentile body mass of white females at 82 kg the daily dosage is 6 X 1600 mg/82 kg = 117.12 mg/kg. The average body mass of BKS.Cg-m+/+Lepr$^{db}$/BomTac mice is ~20 g (0.02 kg) at 9 to 12 weeks of age. The average daily food consumption of an animal of this size is ~ 15 gm / day. Thus, 117.12 mg x 0.02 kg = 2.3424 mg/kg/day of DiaMetrix in 15 gm of food for a final concentration of 156.12 mg DiaMetrix/kg of rodent diet. This diet was prepared by blending the appropriate quantities of DiaMetrix with HFD. An equivalent drug formulation in ND was prepared by using water to soften the food granules reducing them to paste into which the various oral drugs were incorporated by mechanical mixing. Metformin was formulated at 0.12% by weight in the two feed preparations. Actos typical daily dosage is 4 mg/day and the drug was formulated at 0.065 mg/kg in the two diets. Byetta was diluted in PBS to a final concentration of 12.2 ng/mL. The resulting solution was administered by subcutaneous injection (100 μL ) twice per day, once in the morning and once in the afternoon.

**Tissue processing:** At the termination of the experiment, at approximately 60 days, the animals were euthanized and exsanguinated. Whole peripheral blood was collected into BD Vacutainer CPT Cell Preparation Tubes (Beckton Dickinson, Franklin Lakes, NJ) and centrifuged at 1,500 x g at room temperature to isolate plasma according to supplier's protocol. Dissected organs (eyes, kidneys, muscle, pancreas, small and large intestine) were fixed in 10% phosphate buffered formalin (Fisher Scientific, Pittsburgh, PA). Tissue specimens were embedded in paraffin and stained with hematoxyllin and eosin. Tissue images were generated in the Core Optical Imaging Facility, which is supported by University of Texas Health Sciences Center at San Antonio, NIH-NCI P30 CA54174 (CTRC), NIH-NIA P30 AG013319 (Nathan Shock Center) and NIH-NIA P01AG19316.

**Bioassays:** The plasma was analyzed by multiplexed immunoassay for the quantitative determination of 34 cytokines, chemokines and growth factors using Procarta cytokine profiling kits (Affymetrix, Fremont, CA). Advanced glycation end product (AGE) OxiSelect ELISA (#STA-317) was purchased from Cell BioLabs, San Diego, CA. Insulin ELISA kit (rat/mouse, # EZRMI-13K) was obtained from Linco Research, St. Charles, MO. Assay kits for glucose (#K613), pyruvate kinase (#K709), citrate (#K655), HDL and LDL/VLDL cholesterol (#K709), triglycerides (#K622), and Aposensor ADP/ATP ratio were obtained from BioVision (Mountain View, CA). For citrate assay, plasma was deproteinized using a sample preparation kit from BioVision (#K808).

**Statistical analysis:** Continuously distributed outcomes were summarized with the mean and standard deviation. Relative values were graphically represented with a heat map. At each level of diet (normal, high fat), treatment groups were contrasted with regard to the mean using analysis of variance and for each biomarker, pairwise comparisons between treatment groups were corrected for multiple testing using the Tukey method. The Hochberg correction was applied across all biomarkers within each level of diet to correct for multiple testing. Treatment group contrasts with regard to body mass were based on a repeated measures linear model with an autoregressive order 1 covariance assumption in terms of treatment, day, and the treatment by day interaction. All statistical testing was two-sided with a nominal and experiment-wise significance level of 5% using SAS Version 9.2. R was used for graphics.

**Results**

**Body mass:** Animal body mass (g) measured through the course of the study are summarized by treatment group in Figures 1 (normal diet) and 2 (high fat diet). In animals fed a normal diet body masses increased continuously both in control animals and all drug treatment groups. In contrast, body masses of all mice maintained on high fat diet initially increased through first 3-4 weeks then began to gradually decline. The sacrifice time was determined based on animal appearance and performance, especially in the high fat diet mice. Mice in the control and Byetta treatment groups were sacrificed on Day 63. Animals treated with DiaMetrix were terminated on Day 56, while metformin and Actos-treated mice were sacrificed on Day 49 because of their largely compromised performance. There was one premature death in Byetta animals on normal diet and two deaths in DiaMetrix treated animals on high fat diet. There were no significant differences between the curves in either diet.

Treatment group contrasts with DiaMetrix and DiaMetrix contrasts with untreated on mean weight at the end of study were not significant, regardless of diet [Normal diet (Actos: 48.7±4.8, Byetta: 52.8±5.9, metformin: 49.3±3.9, DiaMetrix: 47.3±8.0, untreated: 51.1±2.7) High fat diet (Actos: 52.0±2.3, Byetta: 44.6±2.0, metformin: 49.8±4.0, DiaMetrix: 44.9±4.1, untreated: 42.1±5.9).

**Necropsy:** Control mice on normal diet appeared healthy with no visible wounds or scarring, good skin turgor and normal activity level. Large amounts of fat were seen in viscera. Liver, lungs, kidneys, pancreas, cardiac tissue and intestines were unremarkable. Rear leg muscles

had some atrophy. Control mice on high fat diet appeared unhealthy, lethargic and nonreactive to environmental stimuli. Ecchymosis was noted at the injection sites. Viscera were covered with abundant fat tissue. Pancreas was yellow in color and smaller than on normal diet. The intestines were yellowish and swollen.

Metformin-treated mice on normal diet were healthy with no visible wounds or scarring, had good skin turgor and normal activity level. There were no remarkable differences in comparison with respective control, except for mottled liver observed in most animals. Metformin-treated mice on high fat diet had large skin lesions and extensive hair loss. Skin had good turgor. The animals were lethargic and nonreactive to environmental stimuli. Viscera were yellowish and the liver had deposits of yellow adipose tissue. The pancreases were enlarged. There were diffuse tissue hemorrhages. The animals had poor blood clotting. Several animals had kidney/adrenal tumors.

All Actos-treated mice on normal diet appeared healthy but had decreased activity level. The kidneys looked healthy but were encapsulated in fat and showed hemorrhaged vasculature. Livers were mottled in most animals. Large and small intestines were reddish in color. Mice treated with Actos plus high fat diet were clearly lethargic. A large amount of dense fat was seen within the upper peritoneal and retroperitoneal space. Kidneys were enlarged and encapsulated in adipose tissue. The pancreas was pale and enlarged. The liver had small deposits of yellow adipose tissue. The large and small intestines were yellow and swollen. The animals had some wasting of skeletal muscle and gross steatohepatits evidenced by diffuse yellow spotting. The animals did not bleed easily.

Mice treated with Byetta and maintained on normal diet were less active than the respective controls. Their viscera looked healthy but the kidneys were enlarged and the spleens were necrotic in many cases. The pancreas was enlarged and sclerosed but had normal color and shape. The liver was mottled with fatty deposits. Other tissues were unremarkable. Byetta-treated mice on high fat diet looked unhealthy, lethargic, had ecchymosis at the injection sites, poor skin turgor and multiple head and neck lesions. The majority of animals showed signs of abdominal aortic aneurysm. The kidneys were enlarged and encapsulated in fat with leaky blood vessels. The pancreas was poorly perfused, pale in color, reduced in size and sclerosed. Both the liver and intestines were yellow. The livers had small deposits of fat and the intestines were swollen. The animals had hair loss and edema with some scabbing at injection sites indicative of poor wound healing.

The DiaMetrix treated mice on normal diet had no visible wounds or scarring and displayed good skin turgor. Their activity level was somewhat diminished in comparison with controls. The spleen, intestines, lungs and heart were unremarkable. The kidneys and pancreas were enlarged. The livers were mottled and necrotic with fatty deposits in most animals. Mice on high fat diet that were treated with DiaMetrix looked unhealthy and lethargic with visible ecchymosed tissue along the spine primarily at the injection sites, poor skin turgor and multiple lesions in the head and neck region. Visceral tissue was poorly oxygenated. Most organ appearance was similar to that of DiaMetrix plus normal diet. Some

mice showed distinct signs of abdominal aortic aneurysm. Small and large intestines were yellow and swollen. DiaMetrix treated animals had hair loss and edema but not as extensive as the animals treated with Byetta.

Overall, based on appearance of the animals, drug efficacy followed the order: DiaMetrix>Byetta>Actos>metformin.

**Biomarker differences by treatment group and diet**

Biomarker means varied significantly by treatment group and diet (Tables 1 and 2) and the mean values of most biomarkers were elevated in animals on high fat diet relative to normal diet.

**Cytokines, chemokines, endocrine markers and growth factors**

With the normal diet the mean eotaxin (ng/mL), MCP-1 (pg/mL) and MCP-3 (pg/mL) and M-CSF (pg/mL) were decreased after treatment with DiaMetrix relative to untreated [Eotaxin (DiaMetrix: 2.7±0.5, Untreated: 5.3±1.9; p=0.005), MCP-1 (DiaMetrix: 51.2±16.7, Untreated: 94.6±18.4;p<0.001), MCP-3 (DiaMetrix: 224.1±98.3, Untreated: 526±72.6; p<0.001), M-CSF (DiaMetrix: 5±3.1, Untreated: 9.3±2.4; p=0.04)] and with the high fat diet animals these mean values were decreased after with DiaMetrix but not significantly so.

The mean of IL-4 (pg/mL) was increased after treatment with DiaMetrix relative to untreated with both diets but this increase was significant only with the normal diet [Normal diet (DiaMetrix: 2.6±2.0, Untreated: 0.8±0.5; p=0.04), High Fat Diet (DiaMetrix: 2.0±2.2, Untreated: 0.7±0.6; p=0.22)].

The mean of the growth factors G-CSF, GM-CSF, and TGFβ were significantly decreased in animals treated with DiaMetrix relative to untreated in the high fat diet [Normal diet G-CSF (DiaMetrix: 73.2±87.2, Untreated: 44.3±40.1; p=1.0), GM-CSF (DiaMetrix: 182.4±256.6, Untreated: 226.6±535.2; p=0.98), TGFβ (DiaMetrix: 54.8±25.6, Untreated: 83.6±74.9; p=0.93), High Fat Diet G-CSF (DiaMetrix: 56.2±74, Untreated: 735.8±769.6; p=0.02), GM-CSF (DiaMetrix: 81.9±129.7, Untreated: 457.8±433.1; p=0.03), TGFβ (DiaMetrix: 22.5±34.3, Untreated: 74.9±72.1; p=0.005)].

**Metabolites**

The mean pyruvate kinase (mU/mL) was increased in animals treated with DiaMetrix relative to untreated in both diets, but significantly so with the normal diet [Normal diet (DiaMetrix: 2.1±0.9, Untreated: 0.7±0.9; p=0.02) High Fat Diet (DiaMetrix: 2.0±0.8, Untreated: 1.6±0.8; p=0.94)].

The mean advanced glycation end product (mg/dL) (Figures 3 and 4) was increased after treatment with DiaMetrix relative to untreated with both diets and the increase was significant only with the normal diet [Normal diet (DiaMetrix: 5.4±2.1, Untreated: 1.6±0.6; p<0.001) High Fat Diet (DiaMetrix: 2.4±1.8, Untreated: 1.7±0.8; p=0.90)].

The mean insulin (ng/mL) (Figures 5 and 6) was significantly decreased after treatment with DiaMetrix relative to untreated with the normal diet and non-significantly increased with the high fat diet [Normal diet (DiaMetrix: 4.4±4.9, Untreated: 16.8±6.0; p<0.001) High Fat Diet (DiaMetrix: 3.5±1.3, Untreated: 3.0±4.6; p=0.55)].

The mean citrate (mM) (Figures 7 and 8) was increased after treatment with DiaMetrix with both diets, significantly so with the high fat diet [Normal diet (DiaMetrix: 0.4±0.24, Untreated: 0.21±0.19; p=0.09) High Fat Diet (DiaMetrix: 0.63±0.16, Untreated: 0.22±0.27; p=0.007)].

**Treatment group contrasts**

Treatment group contrasts on biomarker means varied with diet and biomarker. With the normal diet, the mean MCP-3 (pg/mL) was significantly increased after treatment with Actos, Byetta and metformin relative to DiaMetrix [Actos: 460.7±189.9 (p<0.001), Byetta: 476.0±99.9 (p<0.001), metformin: 502.7±175.8 (p<0.001), DiaMetrix: 224.1±98.3]; the corresponding contrasts were not significant with the high fat diet [Actos: 757.8±193.2 (p=0.81), Byetta: 860.5±177.5 (p=0.57), metformin: 457.0±287.7 (p=0.94), DiaMetrix: 455.7±433.1].

With the high fat diet, the mean IL-17 (pg/mL) was decreased after treatment with DiaMetrix relative to Actos, Byetta and metformin, significantly so relative to Byetta [Normal diet, Actos: 66.2±41.5 (p=0.51), Byetta: 133.0±53.5 (p=0.81), metformin: 144.5±127.7 (p=0.91), DiaMetrix: 104.6±54.8, High fat diet, Actos: 439.0±230.0 (p=0.09), Byetta: 953.4±660.5 (p<0.001), metformin: 377.2±181.2 (p=0.18), DiaMetrix: 196.4±159.8].

With the normal diet, the mean advanced glycation end product (mg/dL) was significantly decreased after treatment with Actos and metformin relative to DiaMetrix [Actos: 1.6±0.3 (p<0.001), Byetta: 4.4±1.8 (p=0.79), metformin: 1.9±0.7 (p<0.001), DiaMetrix: 5.4±2.1]; the corresponding contrasts were not significant with the high fat diet [Actos: 2.5±1.9 (p=1.0), Byetta: 2.8±2.5 (p=1.0), metformin: 1.3±0.2 (p=0.64), DiaMetrix: 2.4±1.8].

With the normal diet, the mean insulin (ng/mL) was significantly increased after treatment with Actos, Byetta and metformin relative to DiaMetrix [Actos: 8.8±4.5 (p=0.04), Byetta: 11.6±4.9 (p=0.004), metformin: 20.7±2.5 (p<0.001), DiaMetrix: 4.4±4.9]; the corresponding contrasts were not significant with the high fat diet [Actos: 4.4±3.6 (p=1.0), Byetta: 1.4±0.4 (p=0.73), metformin: 4.6±4.1 (p=1.0), DiaMetrix: 3.5±1.3].

Bivariate clustering by treatment group and biomarker was carried out for each diet and summarized with heat maps (Figures 9 and 10); in these figures high levels of a biomarker are represented in blue, low levels in green and missing values are indicated with white. Comparison of Figure 9 (normal diet) with Figure 10 (high fat diet) shows variation with both biomarker and treatment. For example with normal diet (Figure 9), triglycerides, HDL, advanced glycation end product form a separate cluster but these are not clustered with the high fat diet (Figure 10). In the normal diet (Table 9), but not in the high fat diet (Figure 10), DiaMetrix tends to cluster near untreated.

**Discussion**

The study illustrated the biochemical activity of DiaMetrix relative to the commonly used anti-diabetic drugs against a background of genetic obesity. Analysis of the patterns of cytokine and other biomarkers regulation provided insights into how DiaMetrix exerts its salutary effects in diabetes by significantly regulating expression of a broad range of surrogate markers of diabetic severity.

Using animal performance status as the endpoint we concluded that DiaMetrix and Byetta were better tolerated than metformin and Actos. Increased mortality in the high fat diet of DiaMetrix treated animals did not come as a surprise. Given high concentration of hydroxycitrate in DiaMetrix preparation, feeding the animals with lipids as a primary food source resulted in shutting down the tricarboxylic acid cycle. In fact, this demonstrated the efficacy of DiaMetrix in the experimental diabetes model.

We noted several surprising findings regarding DiaMetrix and other drug effects on select metabolites, in particular glucose, triglycerides, HDL and LDL plus VLDL cholesterol fractions, and ADP and ATP levels, which were not significantly affected by any treatment on normal or high fat diet. These results are likely an outcome of the *in vivo* model system, which shares only some characteristics with human type 2 diabetes (*14*).

Another unexpected finding in this study was lack of significant effect of DiaMetrix on blood glucose levels in contrast to the human study, in which we observed a rapid drop of blood glucose in diabetic patients within 30 minutes of DiaMetrix administration (Hampshire et al, manuscript in preparation). The potent hypoglycemic activity of Syntre-5 is consistent with anti-inflammatory activity of the agent. As pro-inflammatory cytokines increase glucose levels (*15*), other proteins such as TNF$\alpha$ or MIF (*16*) (*17*) can cause rapid drop in blood glucose level within minutes of administration. We need to keep in mind that our animal model of human type 2 diabetes cannot be readily interpolated to human disease. For example, based on known data on blood glucose in normal mice (*18*), we found that the animals at the end of the study were normoglycemic, not hyperglycemic. Hypoglycemia or normoglycemia are not in common in diabetes type 2 (*19*), (*20*) and diet has a large effect on blood glucose levels (*21*). Given major differences in drug treatment (short-term in the clinic versus long term *in vivo*) and limitations of the *in vivo* model, these apparently contradictory findings can be easily reconciled.

Significant inter-group differences in expression levels of several markers were dependent on the diet. Advanced glycation end product (AGE), which is exemplified by glycated hemoglobin (hemoglobin A1c), is formed in a non-enzymatic pathway by exposure of plasma proteins to high levels of glucose (*22*). Protein glycation is associated with cardiovascular disease, nephropathy and retinopathy. Although DiaMetrix on normal diet was associated with increased advanced glycation end product concentrations, a similar effect was also observed for Byetta. Since the levels of advanced glycation end product are proportional not only to concentration of plasma glucose but also to the half-life of target proteins and are genetically determined (*23*), it is conceivable that both DiaMetrix and Byetta might have affected turnover of glycated proteins in this *in vivo* model.

We feel that metrics, such as advanced glycation end product level, should be considered more in the frame of being disease symptoms rather than being elevated to the status of disease and/or mechanisms of disease. These markers should not be seen as levers that can be grasped to alter physiology - they are more like pressure gauges on a steam engine. Blood sugar for instance has been twisted into this status. Many think that by 'controlling' i.e. lowering blood sugar, that the disease is controlled. Yet even with perfect glucose control there are still problems. Drugs that are designed to control blood sugar for instance have many liabilities such as hypoglycemia. Thus the emphasis on controlling blood sugar as a sole endpoint may be misplaced. It is much more important to look at the causes of diabetes, i.e. the immune system. High blood sugar should be seen for what it is - a symptom of disease, not a disease in and of itself. We are not saying, however, that high blood sugar is not a problem, because it is, but it is not the whole problem. Therefore, the cytokine results are extremely important in supporting contentions about the effect of the drug on the disease, not just the symptoms. Given that type 2 diabetes is fundamentally an inflammatory disease, the insights gained may also guide decisions about composition of the drug.

Insulin, which regulates the level of blood glucose, is still produced in the pancreas by the Langerhans isles in type 2 diabetes, however the response of cells throughout the body is abnormal. By reducing the concentration of glucose in the blood, insulin is thought to reduce the long-term complications of diabetes, including damage to the blood vessels, eyes, kidneys and nerves (*24*). In this study, DiaMetrix induced significant hypoinsulinemia on normal diet. This can be viewed as a beneficial effect of DiaMetrix considering that the ob/ob mice with a deficiency in the leptin pathway have elevated insulin levels (*25*), *(26).*

Pyruvate kinase, which regulates the rate-limiting final step of glycolysis, catalyzes conversion of phosphoenolpyruvate to pyruvate, which enters the citric acid cycle after conversion to acetyl-CoA and reaction with oxaloacetate to produce citric acid. In diabetes the rate of glycolysis is decreased and some of that effect is attributable to pyruvate kinase deficiency. The assayable activity of skeletal pyruvate kinase does not always reflect enzyme concentration (*27*). Pyruvate kinase is reversibly inhibited by phosphorylation or ATP alone. In

addition low blood glucose induces phosphorylation. DiaMetrix elevated pyruvate kinase activity on normal diet while other drugs had no significant effects. Increased activity of pyruvate kinase upon treatment with DiaMetrix is unlikely to be due to reduced glucose or ATP levels, as these markers were not significantly different from the control. On the other hand, long term exposure to hydroxycitrate, one of the main ingredients of DiaMetrix, may lead to up-regulation of pyruvate kinase expression. Examples of such drug-induced up-regulation have been reported (*28*).

Citrate is an intermediate in the tricarboxylic acid cycle, the main energy producing mechanism in a cell. After the pyruvate dehydrogenase complex forms acetyl CoA from pyruvate and cofactors (thiamine pyrophosphate, lipoamide, FAD, NAD+ and CoA), citrate synthase catalyzes the condensation of oxaloacetate with acetyl CoA to form citrate. Citrate was significantly increased by DiaMetrix only on high fat diet, consistent with inefficient tricarboxylic acid cycle operation and decreased utilization of citrate.

In the past few years diabetes has come to be increasingly recognized as an inflammatory disease in which obesity plays a key role in the metabolic syndrome related to insulin resistance and heightened risk for the development of type 2 diabetes. Obesity is associated with low-grade chronic inflammation associated with the adipose tissue. In turn, the activated inflammatory cascade cross-talks with insulin signaling pathways through induction of cytokines, chemokines and growth factors (*29*). Circulating biomarkers of the inflammatory pathway, ion cascade, endothelial dysfunction, and procoagulant imbalance may be associated with development of both type 1 and type 2 diabetes (*21)*. The comorbidities of diabetes, such as obesity, insulin resistance, hyperglycemia, hypertension and dyslipidemia, further aggravate the nephropathy, retinopathy and cardiovascular disease while antihyperglycemic therapies relieve these symptoms (*30*), (*31*).

Remarkably, DiaMetrix significantly down-regulated a range of pro-inflammatory chemokines, cytokines, and growth factors. The roles of eotaxin (*32*), MCP-1 (*33-34*), MCP-3 (*35*) and M-CSF (*36-37*) in maintenance of the inflammatory state are well documented. IL-17 is of particular importance, as the IL-23/IL-17 pathway is a key player in chronic inflammation and a number of related diseases recognized as co-morbidities of diabetes (*38*), (*39*). GM-CSF is a major regulatory factor that controls the functions of granulocyte and macrophage lineage populations at all stages of maturation and plays a key role in inflammatory and autoimmune diseases (*40*). TGFβ acting in concert with inflammatory cytokines supports *de novo* differentiation of IL-17-producing T cells (*41*). In addition, DiaMetrix suppressed circulating levels of a number of important pro-inflammatory markers including the chemokine KC that is homologous to human IL-8 (*42*), IL-6 (*43*), IFNγ (*44*) and VEGF (*45*) but also elevated levels of anti-inflammatory cytokine IL-4 (*46-47*), yet another therapeutically significant target. Mice expressing IL-4 in the pancreas are protected from diabetes (*48*), and the administration of recombinant IL-4 prevents diabetes in diabetic mice (*49*).

Macrophage activity is intimately connected to cytokines and pancreatic islets β-cell death in type 2 diabetes. The islets from patients with type 2 diabetes are infiltrated by

immune cells that produce inflammatory factors (IL-6, KC/IL-8, G-CSF, MIP-1α and MCP-1). Increased islet-associated macrophages are observed in human type 2 diabetic patients and in most animal models of diabetes, and in islets of mice on high fat diet even before the onset of diabetes (*37*). DiaMetrix, by virtue of down-regulating relevant inflammatory molecules and up-regulating anti-inflammatory molecules, may break this vicious cycle and slow down the process of islet cell destruction.

Since one of the characteristics of diabetes is derangement of the immune system, and because the markers measured are all immune system messages and effectors, drug-induced differences from control are indicative of drug effects in treatment groups and are directly implicated in the multi-organ dysfunctions characteristic of diabetes. In normal immune function, the inflammatory markers peak first in response to some stimulus, for instance in the case of infection, subsequently the anti-inflammatory markers step in at a later time to limit the damage caused by the pro-inflammatory markers. In this way the immune system mobilizes to first attack an invader and then calls a ceasefire to keep collateral damage down. In many autoimmune diseases, such as diabetes, the linkage and coordination of the pro- and anti-inflammatory mechanisms seems to be broken. For instance both can be elevated simultaneously. This kind of discoordination is common in many immune mediated diseases.

Mechanistically, the functions of cytokines, chemokines and growth factors are reasonably well understood. Many pro-inflammatory factors are viewed as novel therapeutic targets for the treatment of chronic inflammatory diseases, for example IL-6 (*50*), TNFα (*51*), IL-17, GM-CSF (*38-40*). DiaMetrix has been shown to down-regulate the two latter markers. The immune system and its effectors are the primary mediators of cellular and organ damage in diabetes. Thus evaluating the effect of the drug on the immune system will allow us to say with a great deal of confidence how the drug causes its beneficial effect. These results, when considered in light of the results of histological examination, support the contention that DiaMetrix may be an effective intervention for type 2 diabetes.

**References**


1. Kohn, L. D., Wallace, B., Schwartz, F., and McCall, K. (2005) Is type 2 diabetes an autoimmune-inflammatory disorder of the innate immune system?, *Endocrinology 146*, 4189-4191.
2. De Souza, C. T., Araujo, E. P., Bordin, S., Ashimine, R., Zollner, R. L., Boschero, A. C., Saad, M. J., and Velloso, L. A. (2005) Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus, *Endocrinology 146*, 4192-4199.
3. Howard, B. V. (2002) Dietary fat as a risk factor for type 2 diabetes, *Ann N Y Acad Sci 967*, 324-328.
4. Han, D. H., Hansen, P. A., Host, H. H., and Holloszy, J. O. (1997) Insulin resistance of muscle glucose transport in rats fed a high-fat diet: a reevaluation, *Diabetes 46*, 1761-1767.
5. Oakes, N. D., Cooney, G. J., Camilleri, S., Chisholm, D. J., and Kraegen, E. W. (1997) Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding, *Diabetes 46*, 1768-1774.

6. Venables, M. C., and Jeukendrup, A. E. (2009) Physical inactivity and obesity: links with insulin resistance and type 2 diabetes mellitus, *Diabetes Metab Res Rev 25 Suppl 1*, S18-23.
7. Saenz, A., Fernandez-Esteban, I., Mataix, A., Ausejo, M., Roque, M., and Moher, D. (2005) Metformin monotherapy for type 2 diabetes mellitus, *Cochrane Database Syst Rev*, CD002966.
8. Lawrence, J. M., and Reckless, J. P. (2001) Actos (pioglitazone): a new treatment for type 2 diabetes, *Hosp Med 62*, 411-416.
9. Bond, A. (2006) Exenatide (Byetta) as a novel treatment option for type 2 diabetes mellitus, *Proc (Bayl Univ Med Cent) 19*, 281-284.
10. Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L. H., and Hotamisligil, G. S. (2004) Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes, *Science 306*, 457-461.
11. Sharma, K., McCue, P., and Dunn, S. R. (2003) Diabetic kidney disease in the db/db mouse, *Am J Physiol Renal Physiol 284*, F1138-1144.
12. Goncalves, A. C., Tank, J., Diedrich, A., Hilzendeger, A., Plehm, R., Bader, M., Luft, F. C., Jordan, J., and Gross, V. (2009) Diabetic hypertensive leptin receptor-deficient db/db mice develop cardioregulatory autonomic dysfunction, *Hypertension 53*, 387-392.
13. Mastropaolo, M. D., Evans, N. P., Byrnes, M. K., Stevens, A. M., Robertson, J. L., and Melville, S. B. (2005) Synergy in polymicrobial infections in a mouse model of type 2 diabetes, *Infect Immun 73*, 6055-6063.
14. http://www.taconic.com/wmspage.cfm?parm1=16.
15. Kiely, A., McClenaghan, N. H., Flatt, P. R., and Newsholme, P. (2007) Pro-inflammatory cytokines increase glucose, alanine and triacylglycerol utilization but inhibit insulin secretion in a clonal pancreatic beta-cell line, *J Endocrinol 195*, 113-123.
16. Atsumi, T., Cho, Y. R., Leng, L., McDonald, C., Yu, T., Danton, C., Hong, E. G., Mitchell, R. A., Metz, C., Niwa, H., Takeuchi, J., Onodera, S., Umino, T., Yoshioka, N., Koike, T., Kim, J. K., and Bucala, R. (2007) The proinflammatory cytokine macrophage migration inhibitory factor regulates glucose metabolism during systemic inflammation, *J Immunol 179*, 5399-5406.
17. Sanchez-Zamora, Y., Terrazas, L. I., Vilches-Flores, A., Leal, E., Juarez, I., Whitacre, C., Kithcart, A., Pruitt, J., Sielecki, T., Satoskar, A. R., and Rodriguez-Sosa, M. (2010) Macrophage migration inhibitory factor is a therapeutic target in treatment of non-insulin-dependent diabetes mellitus, *FASEB J 24*, 2583-2590.
18. Wong, F. S., and Janeway, C. A., Jr. (1999) Insulin-dependent diabetes mellitus and its animal models, *Curr Opin Immunol 11*, 643-647.
19. Murata, G. H., Duckworth, W. C., Hoffman, R. M., Wendel, C. S., Mohler, M. J., and Shah, J. H. (2004) Hypoglycemia in type 2 diabetes: a critical review, *Biomed Pharmacother 58*, 551-559.
20. DeFronzo, R. A. (2004) Pathogenesis of type 2 diabetes mellitus, *Med Clin North Am 88*, 787-835, ix.
21. http://www.taconic.com.
22. Monnier, V. M., and Cerami, A. (1981) Nonenzymatic browning in vivo: possible process for aging of long-lived proteins, *Science 211*, 491-493.
23. Leslie, R. D., Beyan, H., Sawtell, P., Boehm, B. O., Spector, T. D., and Snieder, H. (2003) Level of an advanced glycated end product is genetically determined: a study of normal twins, *Diabetes 52*, 2441-2444.
24. Lazarus, R., Sparrow, D., and Weiss, S. (1998) Temporal relations between obesity and insulin: longitudinal data from the Normative Aging Study, *Am J Epidemiol 147*, 173-179.
25. Seufert, J., Kieffer, T. J., and Habener, J. F. (1999) Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice, *Proc Natl Acad Sci U S A 96*, 674-679.

26. Ishii, Y., Ohta, T., Sasase, T., Morinaga, H., Hata, T., Miyajima, K., Katusda, Y., Masuyama, T., Shinohara, M., Kakutani, M., and Matsushita, M. (2010) A high-fat diet inhibits the progression of diabetes mellitus in type 2 diabetic rats, *Nutr Res 30*, 483-491.
27. Johnson, M. L., and Veneziale, C. M. (1980) Concentration of skeletal and cardiac muscle pyruvate kinase determined by specific radioimmunoassay, *Biochim Biophys Acta 611*, 127-135.
28. Fatemi, S. H., Reutiman, T. J., Folsom, T. D., Bell, C., Nos, L., Fried, P., Pearce, D. A., Singh, S., Siderovski, D. P., Willard, F. S., and Fukuda, M. (2006) Chronic olanzapine treatment causes differential expression of genes in frontal cortex of rats as revealed by DNA microarray technique, *Neuropsychopharmacology 31*, 1888-1899.
29. Oliver, E., McGillicuddy, F., Phillips, C., Toomey, S., and Roche, H. M. (2010) The role of inflammation and macrophage accumulation in the development of obesity-induced type 2 diabetes mellitus and the possible therapeutic effects of long-chain n-3 PUFA, *Proc Nutr Soc 69*, 232-243.
30. Goldberg, R. B. (2009) Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications, *J Clin Endocrinol Metab 94*, 3171-3182.
31. Kolb, H., and Mandrup-Poulsen, T. (2010) The global diabetes epidemic as a consequence of lifestyle-induced low-grade inflammation, *Diabetologia 53*, 10-20.
32. Crump, M. P., Rajarathnam, K., Kim, K. S., Clark-Lewis, I., and Sykes, B. D. (1998) Solution structure of eotaxin, a chemokine that selectively recruits eosinophils in allergic inflammation, *J Biol Chem 273*, 22471-22479.
33. Lloyd, C. M., Dorf, M. E., Proudfoot, A., Salant, D. J., and Gutierrez-Ramos, J. C. (1997) Role of MCP-1 and RANTES in inflammation and progression to fibrosis during murine crescentic nephritis, *J Leukoc Biol 62*, 676-680.
34. Conti, P., and DiGioacchino, M. (2001) MCP-1 and RANTES are mediators of acute and chronic inflammation, *Allergy Asthma Proc 22*, 133-137.
35. Tsou, C. L., Peters, W., Si, Y., Slaymaker, S., Aslanian, A. M., Weisberg, S. P., Mack, M., and Charo, I. F. (2007) Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites, *J Clin Invest 117*, 902-909.
36. Oren, H., Erbay, A. R., Balci, M., and Cehreli, S. (2007) Role of novel biomarkers of inflammation in patients with stable coronary heart disease, *Angiology 58*, 148-155.
37. Ehses, J. A., Boni-Schnetzler, M., Faulenbach, M., and Donath, M. Y. (2008) Macrophages, cytokines and beta-cell death in Type 2 diabetes, *Biochem Soc Trans 36*, 340-342.
38. Hunter, C. A. (2007) Act1-ivating IL-17 inflammation, *Nat Immunol 8*, 232-234.
39. Iwakura, Y., and Ishigame, H. (2006) The IL-23/IL-17 axis in inflammation, *J Clin Invest 116*, 1218-1222.
40. Hamilton, J. A. (2002) GM-CSF in inflammation and autoimmunity, *Trends Immunol 23*, 403-408.
41. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells, *Immunity 24*, 179-189.
42. Neels, J. G., Badeanlou, L., Hester, K. D., and Samad, F. (2009) Keratinocyte-derived chemokine in obesity: expression, regulation, and role in adipose macrophage infiltration and glucose homeostasis, *J Biol Chem 284*, 20692-20698.
43. Wu, W., Wang, M., Sun, Z., Wang, X., Miao, J., and Zheng, Z. (2010) The predictive value of TNF-alpha and IL-6 and the incidence of macrovascular complications in patients with type 2 diabetes, *Acta Diabetol*.

44. Hayashi, T., Morimoto, M., Iwata, H., and Onodera, T. (1998) Interferon-gamma plays a role in pancreatic islet-cell destruction of reovirus type 2-induced diabetes-like syndrome in DBA/1 suckling mice, *Int J Exp Pathol 79*, 313-320.
45. Wang, J., Xu, X., Elliott, M. H., Zhu, M., and Le, Y. Z. (2010) Muller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage, *Diabetes 59*, 2297-2305.
46. Zaccone, P., Phillips, J., Conget, I., Gomis, R., Haskins, K., Minty, A., Bendtzen, K., Cooke, A., and Nicoletti, F. (1999) Interleukin-13 prevents autoimmune diabetes in NOD mice, *Diabetes 48*, 1522-1528.
47. Marie, C., Pitton, C., Fitting, C., and Cavaillon, J. M. (1996) Regulation by anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGFbeta)of interleukin-8 production by LPS- and/ or TNFalpha-activated human polymorphonuclear cells, *Mediators Inflamm 5*, 334-340.
48. Gallichan, W. S., Balasa, B., Davies, J. D., and Sarvetnick, N. (1999) Pancreatic IL-4 expression results in islet-reactive Th2 cells that inhibit diabetogenic lymphocytes in the nonobese diabetic mouse, *J Immunol 163*, 1696-1703.
49. Tominaga, Y., Nagata, M., Yasuda, H., Okamoto, N., Arisawa, K., Moriyama, H., Miki, M., Yokono, K., and Kasuga, M. (1998) Administration of IL-4 prevents autoimmune diabetes but enhances pancreatic insulitis in NOD mice, *Clin Immunol Immunopathol 86*, 209-218.
50. Ding, C., Cicuttini, F., Li, J., and Jones, G. (2009) Targeting IL-6 in the treatment of inflammatory and autoimmune diseases, *Expert Opin Investig Drugs 18*, 1457-1466.
51. Murdaca, G., Colombo, B. M., and Puppo, F. (2009) Anti-TNF-alpha inhibitors: a new therapeutic approach for inflammatory immune-mediated diseases: an update upon efficacy and adverse events, *Int J Immunopathol Pharmacol 22*, 557-565.

## Abbreviations

| | |
|---|---|
| GM-CSF | granulocyte macrophage-colony stimulating factor |
| HFD | high fat diet |
| ND | normal diet |
| IL | interleukin |
| MCP-1 | monocyte chemoattractant protein 1 |