

Inflammatory Cytokines, Oxidized Low Density Lipoproteins and Glucose Tolerance

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Introduction:

Diabetes, especially type 2, is a growing problem worldwide (1) and its prevalence in the United States is known to be increased in minorities, especially in some Native American populations living in Southwest such as Pima, which is one of the best documented (2,3). Its causative factors were pointed out as both lifestyle-related and hereditary (4, 35, 36). Gene polymorphisms in factors that are known to control glucose metabolism, such as PPAR, PGC, calpain, K ATPase, etc. were found with increased frequency in some minorities (5-7); however the cumulative effect of such genetic variances cannot fully explain the high prevalence of diabetes in such populations. Lifestyle-related factors, such as obesity, sedentary activities, nutrition rich in saturated fats of animal origin and poor in fresh fruits and vegetables that provide most vitamins and antioxidants in a regular diet were proposed as factors (8); however the evidence has consisted mainly from nutritional surveys which offer indirect evidence for the role of diet (9-11). Recent studies point towards oxidative stress (4) and inflammation (12-18) as important processes coexisting with diabetes; however their causative role is not yet widely accepted.

We are considering some of the factors that are thought to contribute to insulin resistance: constitutional - represented by body mass index (BMI), waist circumference, etc -, as well as dietary – content of saturated and trans- fats from daily food intake. Moreover we are looking into differences between inflammatory cytokines IL-1 beta, IL-4, IL-6 and to a lesser extent IL-1 alpha, as well as oxidized Low Density Lipoproteins (oxLDL), triglycerides and asymmetric dimethylarginine between healthy controls and participants with varying degrees of impairment of their glucose metabolism. We have done so through a prospective, cross-sectional study with voluntary participation from both Native Americans and Caucasians at an established community healthcare center in Northeast Arizona. The study was performed between August 2005 – January 2006 after appropriate IRB approval was obtained.

Materials and Methods:

Participants in this study were scheduled patients and walk-ins in the Outpatient Department of a community healthcare center in Northeast Arizona as well as volunteers, regardless of ethnicity. Criteria for inclusion were adults over 18, non-pregnant, not currently on any oral hypoglycemics or insulin. Exclusion criteria were random glucose levels below 100 mg/dL, current treatment with steroids, thiazide diuretics, phenitoin, estrogens, barbiturates, lithium. While waiting for their primary complaints to be heard and addressed by a primary healthcare provider from the clinic, the participants were informed about the study, what it entailed and its benefits. Potential participants were screened for a random blood glucose level by fingerstick with Accucheck or by venipuncture – part of the Chem 7 panel- and those with results above 100 mg/dL were asked to participate in the study. After a short verbal presentation, written information was given about the study – including on contacting The Institutional

Review Board if something is wrong. Those who agreed to participate were scheduled for an appointment – usually within a week- at the clinic for a 2 hour Oral Glucose Tolerance Test after overnight fasting.

On the morning of the first study visit patient's height, weight and waist circumference were measured; after making sure that the patient was fasting we performed the first blood collection by antecubital venipuncture and blood glucose levels were measured from whole venous blood. If the blood glucose level was less than 140 mg/dL the patient was given a standardized drink prepared by Fisher Scientific that contained 75 g of anhydrous glucose with artificial citrus flavor and color. Before completing the next two venipunctures – at 60 minutes and 120 minutes – the patient was given for completion a simple questionnaire on her/his eating habits. We have used 6 questions, both open-ended and with pre-determined choices in order to cover as many aspects of the diet as possible; there was some overlap in some of the questions to try to minimize bias from various backgrounds. The answer to each question was scored with 0, 1 or 2 points according to the implied use of saturated fats: 0 – never/none/very infrequent/very little; 1 – once or twice weekly/some; 2 – frequent/a lot.

After blood collection in the respective Vacutainer tubes – EDTA or no additive – the specimens were further prepared by centrifugation within 15 minutes of collection and the respective plasma or serum was frozen and kept in storage at -30 F until shipment to the laboratories for testing; shipment was done same-day or overnight on dry ice. Using a 7 digit-and-letter code identified the specimens and the correspondence table was kept confidential.

The intervention performed as part of this study consisted in providing each patient with a 14 day supply of dietary supplements consisting of the following daily dosages: 1. essential aminoacids (4 capsules/day with 1 g of protein/capsule from soy and whey hydrolysate manufactured by Optimum Nutrition) 2. polyunsaturated fatty acids softgels; 1 g total – SuperOmega Complex – by Spring Valley: Eicosapentanoic Acid 180 mg; Docosahexanoic Acid 120 mg; Gamma-Linolenic Acid 11 mg; Linoleic Acid 18+18+140 mg, Oleic Acid 7+21+15 mg, Alpha-Linolenic Acid 75 mg, 3. Antioxidant Formula (Vitamin A 10,000 IU, Vitamin C 250 mg, Vitamin E 200 IU, Zinc 7.5 mg, Selenium 15 mcg, Copper 1 mg, Manganese 1.5 mg) 1 softgel – Spring Valley 4. S- adenosylmethionine; 200 mg x 2 tablets – Spring Valley and Nature Made 5. L-Arginine, 500 mg, 1 capsule/day – Nature Made 6. L- Glutamine, 500 mg – Rexall, 1 tablet 7. Ester- C, 500 mg, 2 tablets – Spring Valley. 2 pill organizers were given to each patient with the supplements mentioned above; for most patients those organizers were filled in their presence from the supplement containers and a brief explanation about their role was given and questions were answered. Emphasis was placed on not eating saturated fats for the 2 weeks between the clinic visits; to help enforce the use of unsaturated fats for cooking, each patient was also given 1 container with 2 Quarts of Olive Oil.

The second study visit for each patient was scheduled at 14 days from the first visit, and the patient was again instructed to come at the clinic after fasting overnight for the 2 hr OGTT. We followed the same protocol for the second OGTT; a final questionnaire was given to each patient that included questions on adverse effects observed and concomitant medications used. Additional information was asked from female participants on the timing of their menstrual cycle.

Blood glucose levels were measured in the facility laboratory; the equipment used was tested for accuracy according to CLIA standards. Insulin levels were measured from plasma samples that were kept frozen at -30 degrees and tested at ARUP Laboratories in Salt Lake City, Utah.

To evaluate better the overall status of the glucose metabolism we have used a simple index - GMI (glucose metabolic index) - defined as the area under the curve plotted with the 3 glucose values from the OGTT: fasting (F), 1 hour (H1) and 2 hours (H2); it is calculated with the formula: $GMI = F + (H1 \times 2) + H2$; a graphic representation is provided in **Figure 1**:

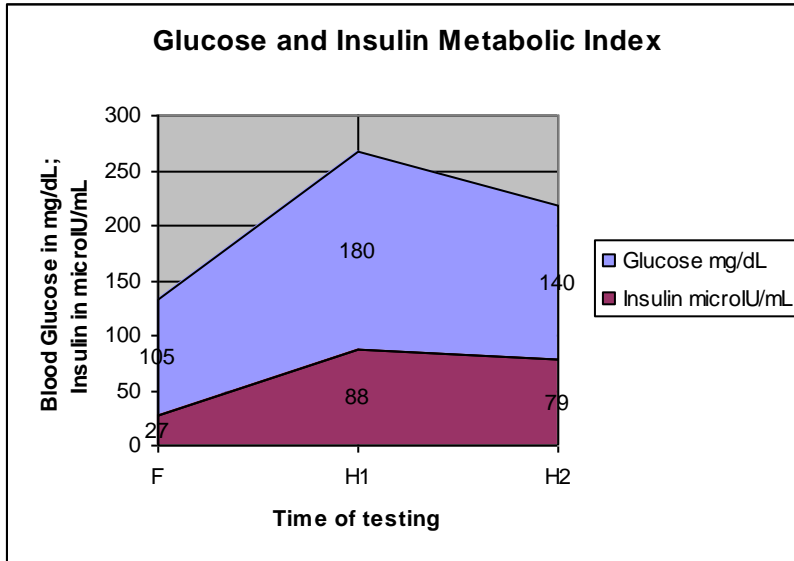


Figure 1: Glucose Metabolic Index (GMI) and Insulin Metabolic Index (IMI)

A Weight Distribution Index (WDI) was calculated by dividing the weight of the patient in lbs by the waist circumference (in inches). Patients with more abdominal fat (truncal obesity pattern) will have a lower WDI than patients with less abdominal bulk. Another index that includes patient's height was calculated by multiplying WDI with patients' height measured in centimeters – we called this Constitutional Index – CI (cm*lbs/in).

We have tested for IL-1B, IL-4, IL-6 and Il-1A in the Research Department of TriCore Laboratories in Albuquerque, NM. Testing was performed with the respective EIA kits from Cayman Chemical (Ann Arbor, MI) and read at 405 nm with the Meridian Premier plate reader. For oxLDL testing we have used first-generation EIA kits from Mercodia (Research Triangle Park, North Carolina) and most of the testing was performed at TriCore Labs Research Department. Additionally 30 specimens were tested at IBT Reference Labs using the same type of Mercodia kits; 5 of these specimens were tested in both labs and yielded similar values; in the analysis we have used the average of those results.

Additionally we have sent some specimens to be tested for Homocysteine, asymmetric dimethylarginine (ADMA) which were done on frozen plasma and a Metabolic Syndrome Panel (insulin, glucose, HDL, triglycerides, eicosapentanoic acid, arachidonic acid) performed from serum that was stored frozen and shipped overnight on dry ice to Metamatrix Laboratories in Norcross, Georgia.

The data obtained from testing was then tabulated and analyzed with Microsoft Excel and NCSS. The study was approved by the corresponding Institutional Review Board and each patient has signed an approved Informed Consent Form after agreeing to participate in the study.

Results:

155 patients were screened for random blood glucose levels; 56 met the enrolment criteria and agreed to participate in the study; of these 45 completed the first OGTT testing and 38 the whole 2-week study; 3 other patients have only done one of the two required OGTT tests. Of the 38 who completed the study, 4 have taken a third OGTT test after 2 more weeks of taking the supplements; we have summarized the demographic data in **Table 1**.

Table 1: Demographics at initial visit:

	Group N	Group IR	Group IR 2	Group IGT	Group D	Total n (%)
Females	6	11	2	6	4	29 (74.4)
Males	3	3	0	2	2	10 (25.6)
Native American	7	14	2	8	6	37 (94.9)
Caucasian	2	0	0	0	0	2 (5.1)

N = group with normal results on OGTT; IR = group with Insulin Resistance; IR2 = group with Insulin Resistance type 2 (higher glucose at 2 hrs than 1 hr); IGT = group with Impaired Glucose Tolerance; D = group with Diabetes

During and after performing the 2-weeks testing, based on data obtained from the patient, computerized records of prescriptions, review of medical chart and discussion with that patients' healthcare provider, 3 patients were excluded from analysis: SA – taking Synthroid 25 mcg/day - ; MH – polypharmacy including Lipitor and diuretic; ST – also polypharmacy including statins and psychoactive meds. Another patient – KM – was considered for the last 2 weeks of testing (out of 4); this patient was receiving a thiazide diuretic for treatment of hypertension during the first 2 weeks of testing.

Based on the results of the OGTT testing, we have grouped the patients in 5 categories: 1. *Normal (N)* – normal blood glucose levels and insulin levels on OGTT; 2. *Insulin Resistant (IR)* - normal glucose levels and elevated insulin levels -; 3. *Insulin Resistant Type 2 (IR2)* with normal glucose levels, elevated insulin levels and inverted shape of the glycemic curve: H2>H1; 4. *Impaired Glucose Tolerance (IGT)*: those with blood glucose > 140 mg/dL at 2 hours and elevated insulin levels; and 5. *Diabetes (D)*; patients with glucose levels >200mg/dL on two distinct measurements (at both H1 and H2 or in both testing days).

All patients with Impaired Fasting Glucose (F>105 mg/dL) also met the criteria for Diabetes, IGT or IR and were not considered as a separate group in our analysis. Finally, even though there were 2 patients showing insufficient pancreatic insulin secretion (by the low levels of insulin compared to their glucose levels), we could not group them together due to different etiologies: one patient with pancreatitis secondary to ethanol abuse had IGT (blood glucose 191 mg/dL at H2) which was normalized after taking the supplements (final H2 = 133 mg/dL), while the other patient had overt diabetes with low insulin values and the OGTT values did not change with taking of the supplements.

The scores on the diet questionnaire along with the constitutional indices are summarized in **Table 2**.

Table 2: Constitutional variables among the groups:

Patient ID	N	IR	D	IGT	IR2
Total Score	6.67	6.778	9	8.5	6.75
Age	40	41.33	45	42.5	48
Height	170	166.4	162	158.2	159
Initial Weight	183	182.6	180	167.2	154
Initial Waist Circumference	40	42.39	42.5	41.92	40
BMI	28.9	29.94	31.2	30.32	27.4
WDI	4.57	4.288	4.21	3.997	3.83
CI	781	715.5	682	633.8	612
Final Weight	189	187.4	178	153.3	160
Final Waist Circumference	41.2	44.1	42.9	40.5	40.5
BMI	28.9	31.87	30.9	29.74	28.8
WDI	4.58	4.238	4.14	3.794	3.94
CI	791	693.2	670	580.3	465
Initial GMI	371	511.9	879	596.3	461
IF	8.4	12.56	24.2	21.5	12
Final GMI	389	479.6	799	646	440
IF	9.57	12.75	21.8	19.67	15.3

WDI – weight distribution index; CI – constitutional index; GMI – glucose metabolic index; IF – level of fasting insulin; BMI – Body Mass Index; Total Score – sum of points on the diet questionnaire

The total score on the questionnaire was significantly higher in the D (diabetes) group, $p = 0.029$, as well as in the IGT group, $p = 0.021$ – when compared to the N (normal) group, indicating that the diet of people with diabetes and IGT contained a significantly higher amount of saturated and or trans-fats when compared to people who have normal glucose metabolism.

Comparing the total scores of the IR and IR2 groups with that of D group was not statistically significant ($p = 0.065$ and $p = 0.056$ respectively), however the difference was sizeable; it is possible that the sample size may have reduced the statistical significance of the difference.

BMI was not statistically different among the 5 groups; however we have found such a difference when using constitutional indices that consider the distribution of fat: WDI and CI. There were significant differences for the WDI and CI between N vs. IR2 ($p = 0.009$ and 0.008 respectively), N vs. IGT ($p=0.022$ and 0.017 respectively) however there was no statistically significant difference between the N vs D ($p=0.092$ and 0.062 respectively) nor N vs IR groups ($p=0.126$ and 0.137 respectively). The only other statistically significant differences between the groups in study involving constitutional indices was found in height: N vs IR2: $p = 0.002$; N vs. IGT: $p = 0.009$ and N vs D: $p = 0.018$, as well as in weight: IR vs. IR2: $p = 0.044$ for initial visit and IR vs. IGT: $p = 0.006$ for the final visit. However, because there were 3 men in the N group compared to 2 in the IR group, 1 in the IGT and D groups and none in the IR2 groups we cannot conclude that constitutional measures play a significant role in the observed differences. Not only the height of men was higher on average and thus introduced its own bias, but also gender differences in themselves are significant in diabetes prevalence (3).

Another variable studied was the body weight, and if there was a correlation of weight modification with the modification of GMI in various patients; no such correlation was observed

in the patients included in the study; however patients who had lost weight in the two weeks were more likely to have an improvement in their glucose metabolic indexes – **Table 3:**

Table 3: – Distribution of weight modification of patients:

Δ Lbs	+4	+3	+2	+1	0	-1	-2	-3	-4	-5	-6	N/R	Total
No of patients	1	1	1	5	9	5	5	2	2	2	3	3	39
Δ GMI	+33	-72	+14	+3	-27	-3	-21	-10	+4	-8	-31		

N/R – not recorded; Δ GMI delta – change in GMI between initial and final visit (mg/dL);
Δ Lbs – change in patients’ weight between initial and final visit (lbs)

There was a significant difference in the correlation of GMI and constitutional indexes between women with reproductive cycle (n=8) and women without (n=21). It is difficult to quantify this effect because the former tended to be also were significantly older (mean age 50.8 vs. 37.4; p = 0.02), however the combined effect of age and diminished estrogen (tended to correlate in a opposite way when compared with both women with reproductive cycle and men.

In all the 5 groups the average GMI is significantly different from the other 4 – **Table 4** – with the highest p value between N vs. IR2: p = 0.009.

The results of the cytokine and oxLDL testing are summarized in **Tables 4, 5 and 6,**

Table 4: Test Results for oxLDL and Metabolic Syndrome Panel:

	Group N, mean +/- SD (n)	Group IR, mean +/- SD (n)	Group IR 2, mean +/- SD (n)	Group IGT, mean +/- SD (n)	Group D, mean +/- SD (n)	T test, lowest p value
Ox LDL, mU/mL	56.35 +/- 15.13 (11)	54.97 +/- 12.54 (12)	50.34 +/- 17.4 (4)	50.6 +/- 6.32 (7)	48.87 +/- 16.07 (8)	N vs D p = 0.160
ADMA nmol/L	201.3 +/- 38.71 (8)	231.1 +/- 62.14 (9)	206.7 +/- 11.55 (3)	236.11 +/- 37.82 (5)	226.7 +/- 33.5 (6)	N vs IGT p = 0.073
Homocysteine nmol/L	N/R	5.4 +/- 1.1 (5)	N/R	5.8 +/- 1.9 (4)	7.2 +/- 1.5 (7)	D vs. IR p = 0.027
HDL mg/dL	48.2 +/- 4.76 (5)	46.8 +/- 8.23 (5)	35.7 +/- 17 (3)	49.5 +/- 10.2 (4)	N/R	IGT vs. IR2 p = 0.014
Triglycerides mg/dL	84.4 +/- 22.8 (5)	199 +/- 84.1 (5)	85 +/- 26.6 (3)	159 +/- 57.59 (4)	N/R	N vs. IR/IGT p = 0.018/0.037
GMI mg/dL	379 +/- 47.4 (8)	491.1 +/- 42.6 (14)	450 +/- 19.4 (4)	592.1 +/- 46.9 (9)	837.7 +/- 117.4 (11)	IR vs. N/IR2 p = 0.002/0.009
Fasting Insulin μIU/mL	8.84 +/- 1.95 (7)	19.2 +/- 8.06 (10)	14.1 +/- 3.52 (4)	16.3 +/- 8.6 (8)	25.6 +/- 11.1 (11)	N vs. IGT p = 0.023 D vs. IGT = 0.031

N/R – not recorded; HDL – high density lipoprotein; GMI – glucose metabolic index; ADMA – asymmetric dimethylarginine, oxLDL – oxidized Low Density Lipoprotein

Table 5: Test Results for Cytokines:

Cytokine (pg/mL)	Group N (n = 7)	Group IR (n =10)	Group IR 2 (n = 5)	Group IGT (n = 4)	Group D (n = 13)	T test lowest p value
IL-1Beta, mean (SD)	3.22 (2.02)	4.3 (2.16)	2.67 (0.81)	2.61 (1.04)	2.54 (0.56)	D vs. IR; p = 0.015 IR2 vs. IR; p = 0.027 IGT vs. IR; p = 0.036
IL-4, mean (SD)	10.16 (5.10)	11.9 (5.92)	34.1 (30.4)	7.83 (2.30)	12.68 (8.63)	D vs. IGT; p = 0.040 IR vs. IGT; p = 0.044
IL-6, mean (SD)	4.9 (2.14)	5.47 (1.72)	7.09 (2.86)	4.34 (2.14)	5.71 (3.52)	N vs. IR2; p = 0.047
*IL-1Alpha, mean (SD)	6.17 (3.10) [2]	11.40 (1.57) [2]			5.86 (1.36) [4]	D vs. IR; p = 0.029

* Interleukin 1 alpha was tested on a smaller number of patients; number of patients tested in each group is given in square brackets

Table 6: Comparison between initial vs final test values in same patient:

	Initial Visit	Follow-up	T test, p value
˘GMI, mean (SD) mg/dL	554.7 +/- 164.3	536.52 +/- 160.32	0.0003
^oxLDL, mean (SD) mU/mL	55.27 +/- 15.31	50.03 +/- 14.61	0.087
IL-1Beta, mean (SD) pg/mL	3.01 +/- 1.39	3.18 +/- 1.63	0.18
IL-4, mean (SD) pg/mL	16.05 +/- 10.53	14.90 +/- 10.65	0.316
IL-6, mean (SD) pg/mL	5.80 +/- 1.59	7.04 +/- 3.46	0.078
*ADMA, mean (SD), nmol/L	222.2 +/- 45.1	205 +/- 28.7	0.09

˘ GMI – Glucose Metabolic Index

^ oxLDL – oxidized Low Density Lipoprotein

* ADMA – asymmetric dimethyl arginine

Along with ADMA, fasting insulin, glucose, HDL, Triglycerides, EPA, Arachidonic Acid, AA:EPA ratio and GMI. For cytokines there were statistically significant differences between some of the groups, however there was not an identifiable pattern (in the manner N<IR<IGT<D or inversely), probable because the IGT group was smaller in number. In triglycerides we have seen a statistically significant difference between the N vs IR and N vs, IGT groups, which is in concordance with the literature. No Metabolic Syndrome Panel and in consequence no triglyceride levels were done in the D group because of budget considerations.

When compared to the initial visit testing, oxLD values were higher on average - **Table 4** however their absolute values were not significantly different (p=0.08). Similarly, when we considered only those patients who had improved their GMI after the 2 weeks (n=16), their average oxLDL were lower after the intervention, but not significantly different (p=0.08). It is possible that after adjusting for age we will see a statistically significant difference, which will be in concordance with results obtained by Kopprasch et al (19-22).

Discussion:

Multiple considerations prompted us to look for non-genetic factors involved in the diabetes endemic in this community. One observation is a historical reference from a 1939 study in which there was only 1 case of diabetes in a Native American population out of 6000 patients treated in that hospital (23); in the year 2000 at the same hospital there are at least 500 patients with diabetes among any 6000 randomly chosen patients. Such hundreds-fold increase in the diabetes prevalence in 3 generations cannot be explained by any genetic transmission; at best an autosomal dominant transmission of diabetes genes – the one with the most aggressive phenotype expression – may have been followed by less than 50 fold increase in the disease prevalence in 3 generations. Another study (11) shows that in Pima Indians, changes in their diet and exercise patterns over time were accompanied by an increase in diabetes prevalence. Also, Pima Indians living in Mexico had lower obesity rates associated with increased physical labor and consumption of less animal fat compared to their counterparts living in Arizona.

The role of inflammation as modulator of blood glucose levels is also well known, only if by the need to adjust insulin doses in diabetes patients with such concurrent pathology.

However, trying to quantify the overall influence of these factors on the ensuing blood glucose levels is extremely difficult, because they act simultaneously and intricately, with overlapping pathways; moreover there are clear genotype and phenotype influences that may dramatically alter an individual's response to the same stimuli and conditions.

With these in mind, we should interpret these results only when taken together with the other measurements undertaken in the other two papers that were written using the results of this diabetes study.

Results on oxLDL show that the lowest values were obtained in the D group, although there were not significantly different from the other groups ($p > 0.1$). Even though this finding was surprising given the literature (19,24-26) which would lead to expect higher oxLDL values on average in the D group, the results may be due to the testing itself, which is very sensitive from a technical standpoint. Lipid oxidation was shown to continue in frozen samples that are kept at temperatures of less than -70 degrees (and our only choice was storage at -30 degrees). This may falsely elevate oxLDL values in samples that were stored longer, which may have been our case given that samples from the D group were collected 4-6 weeks later compared to the N group. This may have been compounded by the use of first-generation EIA kits, the fact that oxLDL testing was the only one performed in two completely different laboratories (IBT and TriCore) and the lack of experience with handling oxLDL testing, which is not a widespread procedure.

The role of estrogen in insulin resistance through its action on the inflammatory pathway is established (27) and may explain the observed differences in our study between women with and without current reproductive cycle.

Finally, there seems to be a strong interdependence between inflammatory factors, oxidative capacity and circulating lipids (4, 28-32) both in their generation and also in the pathways of their action (through NF κ B, IRS-1 and IRS2), and to have a complete picture of their action is probably better to study them at the same time rather than separately, which we have attempted with some degree of success in our pilot study.

Conclusion:

Even as the health status of the general population has improved significantly over the last few decades, as shown by improved life expectancy, declining mortality assigned to cardiovascular disease and recently from malignancies, we are confronted with new healthcare issues that are affecting well-defined populations within the United States and globally. Those issues have called for focused efforts in diagnosing, treating and preventing disease in the populations who are at risk and with the limited success to date of medicine-only interventions, some have suggested a multi-disciplinary approach to those problems.

Going past the debate of whether the causative factors are mostly nature or nurture, the focus of a prompt, efficient intervention should probably be on the factors that we can change, and that reside without doubt in the lifestyle factors, some of which proven to be effective in type 2 diabetes - exercise, weight loss – (33, 34) and some that have been showed to improve the overall health status of a population regardless of ethnicity: improvements in nutrition and sanitation, which are closely related to the socioeconomic status.

IHS has successfully addressed this issue with the creation of the Special Diabetes Clinics that are helping to bridge the gap between medical intervention and everyday lifestyle modifications needed and proven to work in diabetes. As those Clinics deal only with patients diagnosed with the disease, perhaps more can be done towards the prevention of the disease by screening and early intervention on a larger scale. To the best of our knowledge such efforts are underway to assess the efficacy of metformin as a prevention tool; we are proposing to expand those efforts to include dietary supplements and significantly reduce saturated and trans-fat consumption.

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