Association of hypoadiponectinemia with hypoglutathionemia in NAFLD subjects with and without type 2 diabetes

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Abstract. Objective: The aim of this study is to measure the extent of oxidative stress and to see whether it has any correlation to changes in adiponectin levels in NAFLD subjects with and without Type 2 diabetes.

Methods: Subjects recruited from the Chennai Urban Rural Epidemiology Study comprise of 1: Normal Glucose Tolerance (NGT) subjects without NAFLD; 2: NGT with NAFLD; 3: Type 2 Diabetic patients [T2DM] without NAFLD and 4: T2DM with NAFLD. Thiobarbituric acid reactive substances (TBARS), protein carbonyl (PCO), glutathione and adiponectin levels were measured by standard methods. Ultrasound of the liver was used to diagnose NAFLD.

Results: T2DM subjects with NAFLD had significantly (p < 0.001) higher levels of thiobarbituric acid reactive substances (TBARS) and protein carbonyls (PCO) but lower (p < 0.001) GSH/GSSG ratio and adiponectin levels compared to other three groups. The association of hypoadiponectinemia with NAFLD/Type 2 diabetes was significant even after adjusting for age, gender and BMI, but lost when adjusted for parameters of oxidative stress. While palmitate significantly reduced GSH/GSSG ratio in hepatocytes, addition of exogenous recombinant adiponectin restored the GSH/GSSG ratio comparable to those of untreated cells.

Conclusion: There exists an association of hypoglutathionemia and hypoadiponectinemia in subjects with NAFLD and/or T2DM. In addition to the known beneficial effects, our study also exposes the antioxidant nature of adiponectin.

Keywords: Type 2 diabetes, NAFLD, glutathione, adiponectin, oxidative stress

1. Introduction

Non alcoholic fatty liver disease (NAFLD) is known to be associated with insulin resistance and type 2 diabetes [1] and is considered the hepatic manifestation of metabolic syndrome (MS) [2]. Earlier, we have shown that NAFLD is present in a third of urban Asian Indians and that its prevalence increases with increasing severity of glucose intolerance and also in those with MS [3]. The clinicopathology of NAFLD ranges from simple triglyceride accumulation in liver to hepatic steatosis (NASH) cirrhosis, fibrosis and necrosis.

The current understanding of the mechanisms involved in the pathogenesis of NAFLD is based on a multiple hit hypothesis wherein insulin resistance, oxidative stress, cytokines, inflammation and necrosis are thought to play a key role in the progression of the disease [4]. Many experimental models [5] and human studies [6–8] have found a strong association between severity of NASH and degree of oxidative stress. In the recent years, studies throw light on adipose tissue as an endocrine organ secreting various adipocytokines such as adiponectin, leptin, TNF-α and IL-6 which exert a local, systemic and peripheral effect [9]. Earlier, Mohan et al. [10] have shown that a lower adiponectin level...
was associated with the metabolic syndrome, diabetes, insulin resistance and dyslipidemia in Asian Indians.

Adipocytes release the secretory protein adiponectin in a number of different higher-order complexes and these plasma adiponectin complexes have been shown to exhibit distinct biochemical characteristics [11]. The effects of adiponectin do not just correlate with the metabolic disorders but play a direct role in the regulation of glucose and lipid metabolism [12]. Glutathione, a major antioxidant plays a key role in maintaining the redox status of cells, and is critically involved in the protein disulphide bond formation and oligomerization of adiponectin that is essential for its role in conferring insulin sensitivity [13]. Considering the role of liver and fat dysregulation in the natural history of NAFLD and Type 2 diabetes, it is important to study the molecular intricacies of oxidative stress and hypoadiponectinemia in a comprehensive way. Therefore, the aim of this study is to measure the extent of oxidative stress (with special reference to glutathione turnover) and adiponectin levels in NAFLD subjects with and without Type 2 diabetes.

2. Study design and methods

The subjects were recruited from Chennai Urban Rural Epidemiology Study (CURES); the detailed study design of CURES was described elsewhere [14]. The study was approved by the institutional ethical committee and informed consent was obtained from all study subjects. In Phase 5 of CURES, every fourth subject recruited in Phase 3 (n = 588) was invited to our center to undergo ultrasonography of the abdomen thus maintaining the representativeness of the original CURES sampling frame. For the present study, we randomly selected (using computer-generated random numbers) 45 NGT subjects without NAFLD, 35 NGT subjects with NAFLD, 20 T2DM patients without NAFLD and 20 T2DM patients with NAFLD. Subjects on medications for hepatitis B and C, autoimmune hepatitis, primary biliary cirrhosis, HIV infection, etc were excluded for the present study.

All study subjects underwent an oral glucose tolerance test (OGTT) using 75gm glucose load, except self-reported diabetic subjects for whom fasting venous plasma glucose was measured. Diagnosis of diabetes was based on WHO Consulting group criteria, i.e., 2 hour post load plasma glucose (2hr PG) \( \geq 11.1 \) mmol/l or 200 mg/dl and/or fasting plasma glucose (FBS) \( \geq 126 \) mg/dl or self reported diabetic subjects on treatment by a physician. NGT was diagnosed if 2hr PG was \( < 7.8 \) mmol/l or 140 mg/dl and / or FBS \( < 100 \) mg/dl [15]. Insulin resistance was calculated using the homeostasis assessment model (HOMA-IR) using the formula: fasting insulin (\( \mu U/ml \)) fasting glucose (mmol/L)/22.5.

2.1. Anthropometric measurements

Anthropometric measurements including weight, height and waist measurements were obtained using standardized techniques as detailed elsewhere [14]. Height was measured with a tape to the nearest cm. Weight was measured with traditional spring balance that was kept on a firm horizontal surface. Waist was measured using a non-stretchable fibre measure tape. The body mass index (BMI) was calculated using the formula, weight (kg) / height (m²). Blood pressure was recorded in the sitting position in the right arm to the nearest 2 mm Hg with a mercury sphygmomanometer (Diamond Deluxe BP apparatus, Pune, India). Two readings were taken 5 minutes apart and the mean of the two was taken as the blood pressure.

2.2. Biochemical parameters

Fasting plasma glucose (glucose oxidase-peroxidase method), serum cholesterol (cholesterol oxidase-peroxidase-oxidase-amidopyrine method) serum triglycerides (glycerol phosphate oxidase-peroxidase-amidopyrine method) and HDL cholesterol (direct method-polyethylene glycol-pretreated enzymes) were measured using Hitachi-912 Autoanalyser (Hitachi, Mannheim, Germany). The intra and inter assay co-efficient of variation of the above method were 5 and 10%, respectively. Protein Carbonyl content was evaluated by the Friedewald formula. Glycated haemoglobin (HbA1C) was estimated by high-pressure liquid chromatography using the Variant machine (Bio-Rad, Hercules, Calif., USA). The intra and inter assay co-efficient of variation of HbA1C was < 10%. Plasma levels of Malondialdehyde (MDA), a marker of lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) using a fluorescence methodology [16]. Inter- and intra-assay coefficients of variation of the above method were < 5 and 10%, respectively. Protein Carbonyl content was evaluated by the 2,4-dinitrophenylhydrazine (DNPH) assay [17] with an intra and inter-assay coefficients of variation as 2.2 and 2.8%, respectively. Oxidized (GSSG) and reduced glutathione (GSH) was estimated from whole blood ac-
cording to the method of Sampath Kumar et al with necessary modifications [18] with an intra and inter assay coefficient of variation as < 5.5% and < 6%, respectively. Fasting adiponectin levels were measured using radioimmunoassay (Cat. No. HADP-61HK, Linco Research, St Charles, MO, USA). The intra-assay and the inter-assay co-efficient of variation for adiponectin were 3.8% and 7.4%, respectively.

2.3. Assessment of NAFLD

Ultrasonographic examination of liver was performed by an experienced radiologist, using a high-resolution B-mode ultrasonography system (Logic 400; GE, Milwaukee, WI) having an electric linear transducer mid frequency of 3–5 MHz as previously discussed [19,20]. Ultrasounds for all the study subjects were carried out and read by a single radiologist and the radiologist was masked to all clinical and biochemical characteristics of subjects. The scan was done for an average of 20 min and the images obtained were recorded and photographed. Fatty liver was defined as the presence of an ultrasonographic pattern consistent with “bright liver,” with an evidence of increased ultrasonographic contrast between hepatic and renal parenchyma, vessel blurring, and narrowing of the lumen of the hepatic veins in the absence of findings suggestive of chronic liver disease. NAFLD was defined as any degree of fatty liver in the absence of any alcohol intake. Although “social drinking” is unlikely to be associated with NAFLD, due to underreporting of alcohol intake, we chose to take only those subjects who totally denied consuming any alcohol. This ensured that we excluded alcoholic fatty liver disease. NAFLD, if present, was classified based on the severity of fatty liver based on standard criteria [19].

2.4. HepG2 cells culture

Human hepatoblastoma cells (HepG2) cells were obtained from NCCS, Pune. Cells were grown in DMEM with 10% FBS containing 25 mM glucose and 2 mM glutamine at 37°C in 5% CO2 incubator. On attainment of 70% confluency, cells were sub-cultured and cells from 12th passage were taken for all the experiments. In studies with hepatocytes, full-length recombinant human adiponectin (Biovendor, NC, USA) was used. To induct oxidative stress, we have used BSA conjugated palmitic acid. Briefly, 2 ml of 1M palmitate and 8 ml of 14% BSA in DMEM were mixed to constitute 200 mM stock of palmitate conjugated BSA. HepG2 cells were treated/untreated with 500 µM palmitate overnight in serum-deprived medium and incubated with or without recombinant adiponectin (100 ng/ml) for 24 hours and used for the determination of GSH and GSSG levels.

2.5. Statistical analysis

Comparison between groups were performed using one-way ANOVA with \( p < 0.05 \) as the criterion for significance. Pearson correlation was done between variables and the risk factors. Multiple logistic regression analysis was carried out using disease-state (NAFLD/Type 2 diabetes) as dependent variable and adiponectin as independent variable. All analysis was done using windows based SPSS statistical package (version 12.0, Chicago, IL).

3. Results

Table 1 show the clinical and biochemical characteristics of the study subjects. HOMA-IR, FBS and HbA1C were significantly higher in T2DM subjects with and without NAFLD compared to NGT subjects with and without NAFLD (\( p < 0.001 \)). T2DM subjects with NAFLD had a significantly higher level of triglyceride and a lower level of HDL cholesterol compared to NGT without NAFLD (\( p < 0.001 \)).

The mean GSH/GSSG ratio was significantly lower in T2DM patients with NAFLD compared to the other three groups. T2DM patients without NAFLD had significantly lower GSH/GSSG ratio (\( p < 0.001 \)) compared to the NGT subjects with and without NAFLD. The mean GSH/GSSG ratio was significantly lower in NGT subjects with NAFLD (\( p < 0.001 \)) compared to NGT subjects without NAFLD. T2DM patients with NAFLD had significantly higher levels of protein carbonyls (PCO) compared to the other three groups (\( p < 0.001 \)). PCO levels were significantly higher in T2DM patients without NAFLD compared to both the control groups (\( p < 0.001 \)). The mean thiobarbituric acid reactive substances (TBARS) levels were significantly higher in T2DM patients with NAFLD (\( p < 0.001 \)) compared to the other three groups. T2DM patients without NAFLD had significantly higher levels of TBARS (\( p < 0.001 \)) compared to NGT subjects with and without NAFLD. The mean TBARS levels were significantly higher in NGT subjects with NAFLD compared to NGT subjects without NAFLD (\( p < 0.001 \)). Total adiponectin levels were significantly higher in
NGT subjects without NAFLD compared to the other three groups \((p < 0.001)\) (Table 1). Since gender differences in levels of adiponectin are known characteristics, we have analyzed the mean adiponectin levels separately for men versus women in the study subjects. The mean \((\pm SD)\) adiponectin levels \((\mu g/ml)\) in males from NGT without NAFLD, NGT with NAFLD, T2DM without NAFLD and T2DM with NAFLD were \(9.1 \pm 4.7, 6.8 \pm 3.8, 5.9 \pm 2.6,\) and \(4.8 \pm 1.7,\) respectively. Similar adiponectin levels for females were \(11.5 \pm 3.6, 7.8 \pm 4.1, 6.3 \pm 3.6\) and \(7.1 \pm 2.5.\) Although there was a baseline difference in mean adiponectin levels in males versus females, the decrement in adiponectin levels from control state to disease states followed a similar trend emphasizing that difference in adiponectin levels among the study groups were uninfluenced by gender differences.

Table 2 shows the Pearson correlation analysis of adiponectin with oxidative stress markers among subjects with NGT and T2DM patients with and without NAFLD. Adiponectin showed positive correlation with GSH/GSSG ratio in NGT subjects with NAFLD \((p < 0.05).\) This relationship was stronger in T2DM patients without NAFLD and the strongest correlation was seen in T2DM patients with NAFLD \((p < 0.05).\) Adiponectin showed negative correlation with PCO \((p < 0.001)\) and MDA \((p < 0.001)\) in the study subjects and the strongest correlation was seen in T2DM patients with NAFLD compared to other three groups.

Multiple logistic regression analysis was done using disease-state (NAFLD/Type 2 diabetes) as dependent variable and adiponectin as independent variable (Table 2). The negative association of adiponectin levels with NAFLD/Type 2 diabetes was not significant when adip-

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**Table 1**

Clinical and Biochemical characteristics of the study subjects

| Parameters                  | Control subjects without NAFLD \((n = 45)\) | Control subjects with NAFLD \((n = 35)\) | Diabetic subjects without NAFLD \((n = 20)\) | Diabetic subjects with NAFLD \((n = 20)\) |
|-----------------------------|---------------------------------------------|-------------------------------------------|---------------------------------------------|---------------------------------------------|
| Age (years)                 | 41 \(\pm\) 7                              | 44 \(\pm\) 12                             | 54 \(\pm\) 10*#                            | 46 \(\pm\) 8                                |
| Male n (%)                  | 20 (45)                                    | 23 (66)                                  | 13 (65)                                    | 11 (55)                                    |
| Waist (cm)                  | 87 \(\pm\) 8                               | 91 \(\pm\) 9                              | 92 \(\pm\) 9                                | 93 \(\pm\) 8                                |
| BMI (kg/m²)                 | 23.8 \(\pm\) 3.9                           | 24.2 \(\pm\) 3.8                         | 25.0 \(\pm\) 3.3                            | 26.6 \(\pm\) 3.0*                           |
| Systolic blood pressure (mm Hg) | 120 \(\pm\) 16                             | 123 \(\pm\) 15                            | 129 \(\pm\) 15                              | 130 \(\pm\) 21                              |
| Diastolic blood pressure (mm Hg) | 73 \(\pm\) 11                              | 75 \(\pm\) 10                             | 81 \(\pm\) 9                                | 82 \(\pm\) 11                               |
| HOMA-IR                     | 1.07 \(\pm\) 1.0                           | 1.9 \(\pm\) 1.2                           | 3.3 \(\pm\) 1.6*#                          | 3.8 \(\pm\) 2.3*#                          |
| Fasting plasma glucose (mg/dl) | 97 \(\pm\) 29                              | 101 \(\pm\) 16                            | 168 \(\pm\) 51*#                           | 153 \(\pm\) 57*#                           |
| HbA1c (%)                   | 5.9 \(\pm\) 1.4                             | 6.0 \(\pm\) 1.3                           | 7.9 \(\pm\) 1.9*#                          | 8.9 \(\pm\) 1.6*#                          |
| Serum cholesterol (mg/dl)   | 177 \(\pm\) 41                             | 183 \(\pm\) 33                            | 178 \(\pm\) 40                              | 191 \(\pm\) 40                              |
| Serum triglycerides (mg/dl)  | 110 \(\pm\) 50                             | 137 \(\pm\) 79                            | 156 \(\pm\) 77                              | 189 \(\pm\) 108*                           |
| HDL cholesterol (mg/dl)     | 46 \(\pm\) 12                              | 40 \(\pm\) 9                               | 41 \(\pm\) 14                                | 36 \(\pm\) 7*                              |
| LDL cholesterol (mg/dl)     | 104 \(\pm\) 33                             | 109 \(\pm\) 25                            | 115 \(\pm\) 39                              | 119 \(\pm\) 39                              |
| Adiponectin (µg/ml)         | 10.7 \(\pm\) 4.1                           | 7.0 \(\pm\) 3.9*                          | 6.4 \(\pm\) 2.8*                           | 6.0 \(\pm\) 2.9*                           |
| PCO (nM/mg protein)         | 0.42 \(\pm\) 0.22                          | 0.7 \(\pm\) 0.3*                          | 0.9 \(\pm\) 0.12*#                         | 1.0 \(\pm\) 0.19*#                         |
| MDA (TBARS) (nM/ml)         | 5.4 \(\pm\) 2.4                            | 8.1 \(\pm\) 3.1*                          | 15.0 \(\pm\) 2.6*#                         | 17.0 \(\pm\) 4.3*#                         |
| GSH/GSSG ratio              | 23.7 \(\pm\) 5.0                           | 17.6 \(\pm\) 7.2*                         | 6.1 \(\pm\) 2.0*#                          | 4.1 \(\pm\) 2.0*#                          |

\*\(p < 0.05\) compared to control subjects without NAFLD, \# \(p < 0.05\) compared to control subjects with NAFLD, \% \(p < 0.05\) compared to diabetic subjects without NAFLD; ^ \(p \text{ for trend} < 0.001.\)
4. Discussion

The major findings of this study are: 1. Adiponectin levels showed positive correlation with GSH/GSSG ratio and negatively correlated with TBARS and PCO levels in NAFLD subjects and diabetic patients without and with NAFLD. 2. The association of hypoadiponectinemia with NAFLD/Type 2 diabetes was sta-

![Fig. 1. (a) Correlation of mean adiponectin levels with GSH/GSSG ratio. (b) Correlation of mean adiponectin levels with MDA.](image-url)
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Fig. 2. Antioxidant activity of adiponectin in hepatocytes: Hepatocytes (HepG2) cells were treated with palmitate (500 uM) to impose oxidative stress in the presence and absence of recombinant adiponectin (100 ng/ml) and processed for GSH (Fig. 2a), GSSG (Fig. 2b) and GSH/GSSG estimations (Fig. 2c).

1. Untreated
2. Palmitate
3. Adiponectin
4. Palmitate + Adiponectin

*p<0.05 compared to untreated cells
#p<0.05 compared to palmitate

Antioxidant activity of adiponectin in hepatocytes: Hepatocytes (HepG2) cells were treated with palmitate (500 uM) to impose oxidative stress in the presence and absence of recombinant adiponectin (100 ng/ml) and processed for GSH (Fig. 2a), GSSG (Fig. 2b) and GSH/GSSG estimations (Fig. 2c).

Adipocytokines and oxidative stress are known to play a key role in the pathogenesis of NAFLD and Type 2 diabetes. Circulating levels of adiponectin are an important marker for metabolic disorders [26,27] and are known to elicit direct effect on glucose and lipid metabolism [28]. Several animal models of alcoholic fatty liver have displayed reduced circulating adiponectin levels, decreased hepatic adiponectin receptors expression, and impaired hepatic adiponectin-mediated signaling [29]. Adiponectin exerts its protective effects against alcoholic liver steatosis through interacting with hepatic AdipoR1/R2 and mediating multiple signaling pathways, which eventually lowers lipid accumulation in liver. Studies have shown an increase in oxidative stress, imbalance in pro and antioxidant enzymes and reduced adiponectin levels in NAFLD animal models [30]. Serum adiponectin levels are significantly lower in NAFLD patients with higher grades of inflammation [31]. Mohan et al. [10] have shown that a lower adiponectin level was associated with the metabolic syndrome, diabetes, insulin resistance and dyslipidemia in Asian Indians. On the other hand, increased oxidative stress has been shown associated with impaired glucose tolerance, type 2 diabetes and its microangiopathies [32–35]. In this context, our study emphasizes an association of increased oxidative stress...
with hypoadiponectinemia in NAFLD subjects without and with Type 2 diabetes.

Oxidative stress, a key factor in the pathogenesis of NAFLD was also proposed as the common denominator in different pathways in the pathogenesis of diabetes and its complications [32,36]. Redox status of the cells is crucial for the maintenance of the biological activity of the cellular proteins and structural changes in proteins are considered to be among the molecular mechanisms that results in dysregulation in glucose and lipid homeostasis [37]. GSH is one of the major cellular antioxidants and GSH depletion has been reported in NAFLD [38] and Type 2 diabetes [18]. Functional failure in glutathione turnover as a sequel of an altered GST genotype was expected to aggravate NAFLD [39]. Maintaining the ratio of reduced to oxidized glutathione is very crucial for maintaining normal protein processing and folding and maintaining the biological activity of certain key insulin sensitizing proteins [37]. Adiponectin is an important adipocytokine hormone which circulates in blood as homo-oligomers (trimer, hexamer and high molecular weight (HMW) forms) as well as a truncated form corresponding to the globular domain. The stability and secretion of adiponectin are also regulated at the post-translational modification level via hydroxylolation, glycosylation and disulfide bond formation [40]. It is proposed that redox dependent environment is crucial for adiponectin oligomerization [41]. Adiponectin oligomerization occurs in an oxidized environment in the lumen of endoplasmic reticulum through thiol mediated protein retention [42]. Oxidized glutathione is required for this processing and the level of oxidized GSSG depends on the amount of GSH entering from the cytosol. Therefore, hypoglutathionemia observed in our study might have repercussions on processing of adiponectin, its secretion and biological activity. On the other hand, protein glutathionylation occurs as a response to oxidative stress, where an increased concentration of oxidized glutathione modifies post-translational proteins by thiol disulfide exchange. While one of our earlier studies has demonstrated increased protein glutathionylation in patients with Type 2 diabetes [18], protein glutathionylation was also shown increased in livers with NAFLD [43]. Therefore, the association of adiponectin with GSH/GSSG ratio in our study assumes importance as it not only denotes increased oxidative stress status in NAFLD/Type 2 diabetes but also emphasizes the interrelationship between glutathione levels and its possible involvement in oligomer formation of adiponectin. In this context, the association of hypoglutathionemia and hypoadiponectinemia seen in our study in NAFLD subjects without and with Type 2 diabetes is an important observation.

Cross-sectional study is one of the limitations of our work and hence it may not reflect causal relationship of hypoadiponectinemia and hypoglutathionemia. However, this study is unique in that subjects were recruited from a population-based study and careful inclusion criteria were used in the selection and characterization of the study subjects. Moreover, the in vitro study performed with hepatocytes provide evidence that palmitate-induced alterations in glutathione turnover can be reversed by adiponectin. In fact, adiponectin was shown to protect against endothelial dysfunction and cellular disruption induced by oxLDL through a mechanism of maintenance of intracellular GSH levels [44]. A variant of the gene encoding adiponectin (ADIPOQ) was also associated with variation in plasma total antioxidant status and levels of oxidized low-density lipoprotein (oxLDL) in people with Type 2 diabetes, indicating an antioxidant role for adiponectin [45]. Also, glutathione depletion via buthionine sulfoximine (BSO) administration affected endothelial function and caused hypoadiponectinemia in rats [46]. In an elegant animal model study Fukushima et al. [47] showed that lack of adiponectin enhanced, and adiponectin administration prevented steatohepatitis progression in mice and these changes were due to the anti-oxidative effects of adiponectin. Moreover, regulation of oxidative stress and inflammation by hepatic adiponectin receptor 2 has been recently reported in an animal model of nonalcoholic steatohepatitis [48]. While pioglitazone treatment increases adiponectin levels and is critical to reverse insulin resistance and improve liver histology in NASH patients [49], adiponectin expression was also shown induced by vitamin E via a peroxisome proliferator-activated receptor gamma-dependent mechanism [50]. Very recently, Detopoulou et al. [51] have reported a positive association between diets with high antioxidant capacity and adiponectin levels which emphasize an adiponectin-mediated route through which antioxidant-rich foods are expected to exert beneficial effects. Therefore, in addition to the insulin-sensitizing and anti-inflammatory activity, our study exposes the antioxidant activity of adiponectin.

To conclude, CURES gave us an opportunity to look at the association of hypoadiponectinemia and oxidative stress in NAFLD subjects without and with Type 2 diabetes in an epidemiological setting in Asian Indians. Our study is the first of its kind to look at
the association of hypoadiponectinemia and hypoglutathionemia and warrants the need for further studies that would address the precise mechanisms of interrelationship of hypoadiponectinemia and hypoglutathionemia which would aid in newer therapeutic measures for both NAFLD and Type 2 diabetes.

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