Addis Ababa University

School of Graduate studies

MSc Research Thesis

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Title of Thesis:

Therapeutic benefits of vitamin A for Type 1 Diabetics: A case of Gondar University Hospital Diabetic Center

Sponsors:

University of Gondar

Addis Ababa University

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List of Abbreviations

13cRA=13-cis retinoic acid

9cRA= 9-cis retinoic acid

ADA=American Diabetic Association

ADH= alcohol dehydrogenases and

ATRA= All trans retinoic acid

BMI= Body mass index

CE =Cholesterol esterase

CHD=Coronary heart disease

CHOD= Cholesterol Oxidase

DCCT=Diabetes Control and Complications Trial

DCs=dendritic cells

G6PDH = Glucose-6-phosphate Dehydrogenase

HbA1c= Glycated hemoglobin

HK= hexokinase

IDF=International Diabetes Federation

NOD mice= Non-obese diabetic mice

POD =Peroxidase.

RA= Retinoic acid

RALDH=retinaldehyde dehydrogenase

RAR= Retinoic acid receptor

RAREs=retinoic acid response elements

RXR=Retinoid x receptor

SGOT=Serum glutamate oxaloacetate transaminase

SGPT= Serum glutamate pyruvate transaminase

STZ=streptozotocin

T1DM= Type 1 Diabetes Mellitus

T2DM= type 2 Diabetes Mellitus

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Abstract

The incidence of diabetes mellitus has soared to epidemic proportions worldwide. The debilitating chronic hyperglycemia is caused by either lack of insulin as in diabetes type 1 or its ineffectiveness as in diabetes type 2. Frequent replacement of insulin with or without insulin analogs for optimum glycemic control is the conventional cumbersome therapy. The quest to find a cure for diabetes has led scientists to think new therapeutic strategies like re-educating the immune system, β-cell transplantation and rejuvenating β-cells of the pancreas in an attempt to restore β-cell function and resume physiologic glucose homeostasis.

There are in vitro and animal studies suggesting that Retinoic acid (Vitamin A) induces differentiation of pancreatic ductal epithelial cells to insulin secreting cells and also plays an immunomodulatory role in the progression of the autoimmune process in Type 1 diabetes. Moreover, majority of diabetic patients have Vitamin A deficiency. However, there was inadequate information regarding in vivo effects of retinoic acid in diabetic patients and hence the need for this study.

This study was an interventional study on 24 Type 1 Diabetic patients attending the chronic illness clinic at Gondar University Hospital. The patients were randomly divided in to two groups; treatment and control. The treatment group received vitamin A supplementation in addition to the conventional insulin therapy where as the control group continued with their conventional insulin therapy alone. Both groups were followed for 6 weeks and treatment effect compared across them in terms of glycemic control, C-peptide levels and lipid profile over the treatment period and both groups were comparable with regard to Age, sex, BMI, duration of disease, dosage of insulin therapy and other baseline biochemical parameters.

Results showed a remarkable clinical benefit in the vitamin A treated group with HbA1c levels of 6.7% versus 8.44% for the control group though it was not statistically significant(P = 0.165). Furthermore, the mean C-peptide level among the treated group was raised to 1.06ng/ml from a baseline level of 0.48ng/ml (a 55% increase in endogenous insulin secretion) indicating the potential therapeutic benefits of vitamin A supplementation in Type 1 diabetics and obviating the need for further research preferably using a bigger sample size and a well-designed clinical trial. Besides, Vitamin A could protect or delay the onset of diabetes in high risk groups.

1. INTRODUCTION

The incidence of both Type 1 (T1DM) and Type 2 (T2DM) diabetes is increasing worldwide. Both disorders are characterized by high concentrations of blood glucose (hyperglycemia) and complicated metabolic dysregulation - avoidable by appropriate secretion of insulin by the pancreatic β-cells. The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with diabetes and on the health care system.

From a worldwide perspective, the total number of people with diabetes is expected to rise from 171 million in 2000 to 366 million by 2030[1]. However, the current estimate for 2010 is 285 million adults (aged 20-79 years) with diabetes rising to an estimated 439 million adults with diabetes by 2030 which is 20% higher than the previous estimate for 2030[2]. According to reports from the International Diabetes Federation (IDF) in 2009, the projected rise in diabetes for sub-Saharan Africa is 98%, i.e. from 12.1 million in 2010 to 23.9 million in 2030.This calls for the identification and implementation of affordable preventive measures as well as alternative and/or innovative therapeutic approaches.

Formal estimate of the IDF shows that currently there are more than 4.5 million adults in Ethiopia who have diabetes or prediabetes (11.7% of adults) and the prevalence of diabetes in Ethiopia in 2011 in those aged 20-79 years is 3.5% where as Prevalence of Impaired Glucose Tolerance (Prediabetes) is 8.2%[3].

Type 1 diabetes compromises an individual's insulin production through the selective autoimmune destruction of pancreatic *β*-cells [4]. Type 1 diabetes is usually diagnosed during childhood or adolescence, and is often signaled by a metabolic crisis (diabetic ketoacidosis) triggered by excessively high blood glucose levels [5]. In addition, type 1 diabetes is quite heterogeneous in its presentation, form, and characteristics when examined from either a metabolic or an immunologic perspective [6]. Although much is understood about the mechanisms of this disease, multiple potential contributing factors, both genetic and environmental, are thought to play distinct parts in triggering type1 diabetes. Of those thought to be environmental in nature, vitamins have gained particular attention of late especially vitamins A and D [7].

Insulin has been in general use in treatment of diabetes with insulin deficiency since its discovery. However, normal pancreatic β-cells continually adjust insulin secretion in response to varying blood glucose levels, while daily exogenous insulin administration cannot maintain blood glucose levels within the narrow physiological range that protects diabetics from the development of the various diabetic complications, and more precise administration is impracticable. Thus, new treatment approaches aimed at replacing or regenerating β-cells are gaining momentum in the scientific community as the quest for a cure for diabetes continues. Islet cell transplantation and ways of stimulating transdifferentiation of pancreatic acinar cells in to insulin secreting β-cells have also been shown in *invitro* studies [8-10].

The two major forms of diabetes (T1DM and T2DM) are characterized by pancreatic islet β-cell dysfunction and decreased β-cell numbers, raising hope for cell replacement therapy. Although human islet transplantation is a cell-based therapy under clinical investigation for the treatment of type1 diabetes, the limited availability of human cadaveric islets for transplantation will preclude its widespread therapeutic application. The result has been an intense focus on the development of alternate sources of β-cells, such as through the guided differentiation of stem or precursor cell populations or the transdifferentiation of more plentiful mature cell populations [11].

The β-cells are the major constituents of the islets of Langerhans: a composition of several endocrine cell-types that make up 1–2% of the adult pancreas amongst the more prevalent exocrine and ductal components. T1DM is mainly due to absence of functional β-cells that may associate autoimmune disturbance and genetic susceptibility. While the number of T1DM susceptibility genes and loci is numerous, an even larger list of environmental agents has long been noted to influence - in either a positive or negative fashion. Subnormal islet mass to body weight ratio is also the major cause of T2DM.

Since β-cells are non-proliferating cells in most cases, there should be a renewable source for new β-cells. This source is now established to be the pancreatic ductal cells as the mother multipotent cells of the pancreas. The process of conversion of ductal short columnar cells into endocrine cells is called islet neogenesis or transdifferentiation [12].

1.1. ISLET NEOGENESIS

Islet neogenesis defined as budding of hormone-positive cells from the ductal epithelium, is considered to be one mechanism for normal islet growth after birth and in regeneration, and has suggested the presence of pancreatic stem cells. Numerous results support the neogenesis hypothesis; the evidence for the hypothesis in the adult comes primarily from morphological studies that have in common the production of damage to all or part of the pancreas, with consequent inflammation and repair.

Although numerous studies support a ductal origin for new islets after birth, lineage-tracing experiments are considered the "gold standard" of proof. Lineage-tracing experiments showed that pancreatic duct cells act as progenitors, giving rise to new islets after birth and after injury. The identification of differentiated pancreatic ductal cells as an *in vivo* progenitor for pancreatic β-cells has implications for a potentially important, expandable source of new islets for diabetic replenishment therapy [13].

This process of islet neogenesis is halted in T1DM along with the dismissal of the new islet cells by the immune attack [14-21].

Much interest and hope is being put into research on re-generation of the β-cell mass for patients suffering from diabetes. So far, numerous different paths have been followed to generate new β-cells, and it is difficult to predict which of these are the most promising or which are doomed to fail.

In general, the attempts to restore the original β-cell population can be subdivided into two main categories: the *in vitro* generation of new β-like cells that can be used for transplantation and the *in vivo* regeneration of β-cells in the diabetic pancreas, which could be stimulated by pharmacological agents [12].

1.2. VITAMIN A AND RETINOIDS

A group of natural chemicals called retinoids include naturally occurring vitamin A and its body metabolites (All-trans retinoic acid and 9-cis retinoic acid) as well as synthetic analogs. They are physiological regulators of a number of essential biological processes, such as embryonic development, reproduction, metabolism, proliferation and apoptosis but most importantly cellular differentiation and transdifferentiation [22].

Retinoids are prototypical differentiation agents that have critical roles in the normal cellular functions and differentiation. As such, they have a fundamental role in the chemoprevention and treatment of epithelial tumours. In carcinoma, retinoids may have a role in chemoprevention by inhibiting the proliferation of cancer cells inducing differentiation or apoptosis [15].

Retinoids are also used clinically for the treatment of a number of dermatologic, hematopoietic diseases and cancer. Retinol is alcohol form and retinal is aldehyde form of vitamin A – both are functional in mechanisms of vision. However, the most physiologically important form of vitamin A is its acid form called retinoic acid (with three major natural isomers, all-*trans* retinoic acid (ATRA) and 9-*cis* and 13-*cis* retinoic acid (9cRA and 13cRA). Most of the effects of retinoids are mediated by *retinoic acid receptors* (RARs), which bind both ATRA and 9cRA, and the *retinoid x receptors* (RXRs), which bind 9-*cis* retinoic acid specifically.

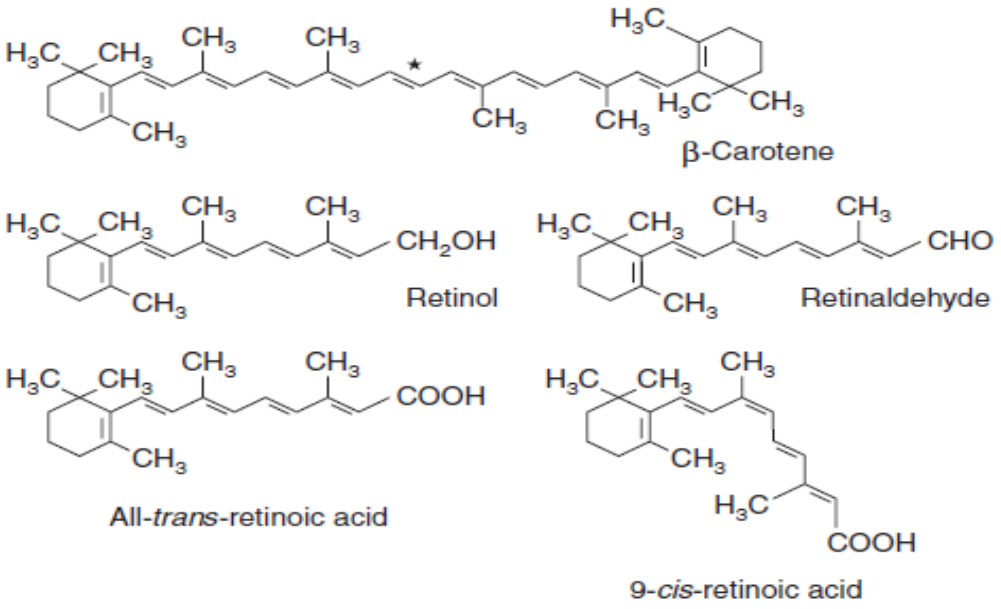

Fig.1.1. Structure of β-carotene and the different retinoids. Beta-carotene from plants is cleaved in the intestinal mucosa by *carotene mono-oxygenase, yielding retinaldehyde

Vitamin A has an impact on a wide range of biological functions such as growth, differentiation, reproduction and vision. Various studies suggest important roles for vitamin A and retinoids in islet cell function. Repletion of Vitamin A deficient rats with Vitamin A or all-trans retinoic acid (ATRA) improves their capability for secreting insulin. At cellular level Vitamin A is converted to ATRA which then isomerizes to 9cRA, another active metabolite of Vitamin A. Both 9cRA and ATRA increased insulin release [23,24].

1.3 METABOLISM AND BASIC ACTIONS OF RETINOIC ACID

Retinol is absorbed into epithelial cells after hydrolysis from its precursor form, retinyl Palmitate. Beta-Carotene, also called provitamin A, is converted to retinal by carotene monooxygenase-1 in epithelial cells [25].

Retinol is esterified and then packaged into chylomicrons in enterocytes for transportation to the liver. It is esterified again and stored in the liver stellate cells and also in fat cells (adipocytes). Retinol is metabolized into retinal and then to biologically active retinoic acids (RAs) by sequential action of alcohol dehydrogenases (ADHs) and retinaldehyde dehydrogenase (RALDH). Retinaldehyde is metabolized into retinoic acid (ATRA) by retinaldehyde dehydrogenases (RALDH).There are three types of RALDH proteins encoded by different genes (i.e. RALDH1, 2, and 3) and with different patterns of expression. Intestinal epithelial cells and dendritic cells constitutively express RALDH to produce ATRA.

All trans retinoic acid and 9cRA activate RAR–RXR heterodimers which specifically bind retinoic acid response elements (RAREs) on chromosomes for regulation of gene expression. Retinoic acid (RA) produced at high levels in the intestine plays important roles in mucosal immunity and immune tolerance. RA at basal levels is required for immune cell survival and activation.

Besides, during immune responses, enzymes metabolizing vitamin A are induced in certain types of immune cells such as dendritic cells [26,27]. Both vitamin A and ATRA have important immune modulatory functions .In the animal models of type 1 diabetes, the NOD mouse and the BB rat, immunomodulatory therapy can alter or block the autoimmune disease process and development of diabetes can be slowed or prevented [28]. Retinoic acid steers the differentiation of T and B cells in the direction to promote both immunity and immune tolerance [29].

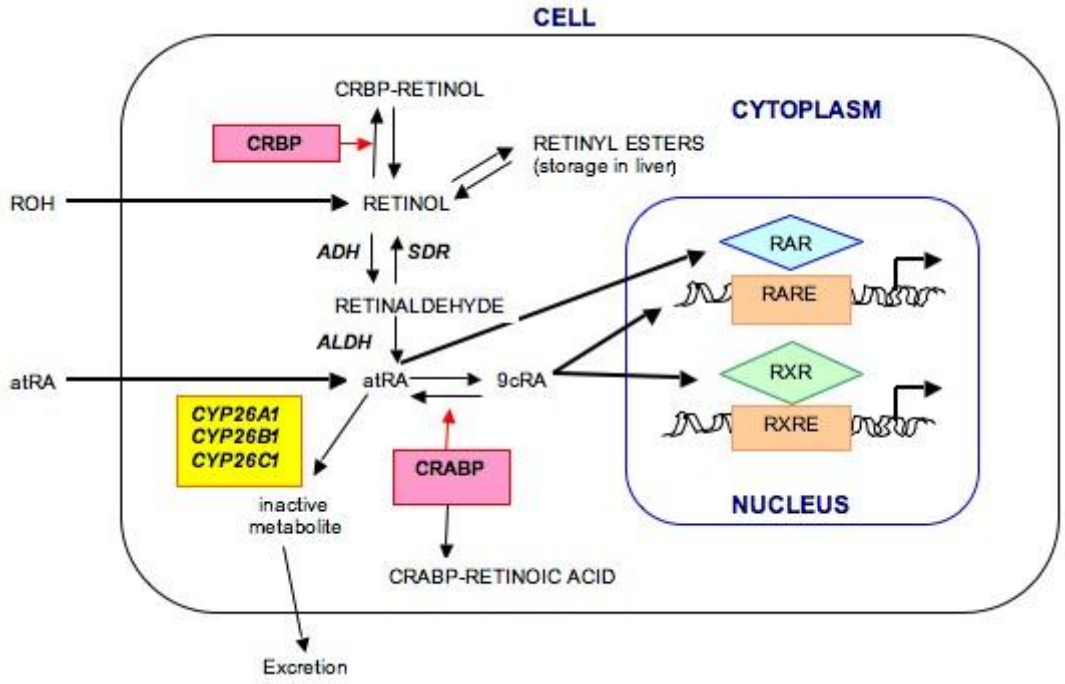

Fig.1.2. Metabolism and actions of vitamin A. CRBP= cytosolic retinol binding protein, CRABP= cytosolic retinoic acid binding protien.

Retinoic acid has manifold effects on pancreatic β-cells. Experimental evidence supports a role for retinoids in the regulation of glucose metabolism and adipose tissue physiology through increasing sensitivity to insulin action [30]. Retinoic acid demonstrates insulinotropic factor in adult pancreas. Considering the role of retinoids as an insulinotropic factor, retinoic acid deficiency was also related to the cause of T1DM. Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into β-Cells [31].

The loss of β-cells that characterizes Type 1 diabetes reflects the net effects of destruction and regeneration. These processes have been examined in non-obese diabetic (NOD) mouse; uncertainty remains about β-cell dynamics in humans. Islet inflammation stimulates beta-cell replication that produces new insulin-positive cells. The regenerative process may tide the loss of overall β-cell function. Residual β-cells play significant role for designing therapeutic trials. They may respond not only to stimulants of metabolic function like Retinoic acid but are also the potential source of new β-cells [32].

Vitamin A and retinoic acid, with immunomodulatory effects, have been ascribed as being relatively deficient in subjects with established T1DM [33]. Vitamin A deficiency can also lead to exacerbation of experimental autoimmune colitis [34]. Vitamin A modulates the adaptive and innate immune systems. Such immune modulation by vitamin A and retinoic acid could decrease the severity of autoimmune diseases. This would protect the newly born islet cells from clearance by immune attack [6, 32, 35]. This is mainly through modulation of the expression of specific cytokines and the membrane mucins [14].

All trans retinoic acid (ATRA) treatment inhibited diabetes in NOD mice with established insulitis and also promoted in vivo expansion of Treg cells and induced Treg cell–dependent immune tolerance by suppressing IFN-γ–producing T-cells, without affecting Th17 cells [36,37]. Supplementation of vitamin A to animals results in a decrease in serum pro-inflammatory cytokines, including tumor necrosis factor-α and IFN-γ, and an increase in the immunosuppressive cytokine IL-10 [38,39].

Indeed, recent studies have noted vitamin A as a major "agent of influence" in the development of what is widely referred to as "oral tolerance" to dietary agents, as well as in the regulation of immune responses, in general, [29] as shown in Fig. 1.3 below.

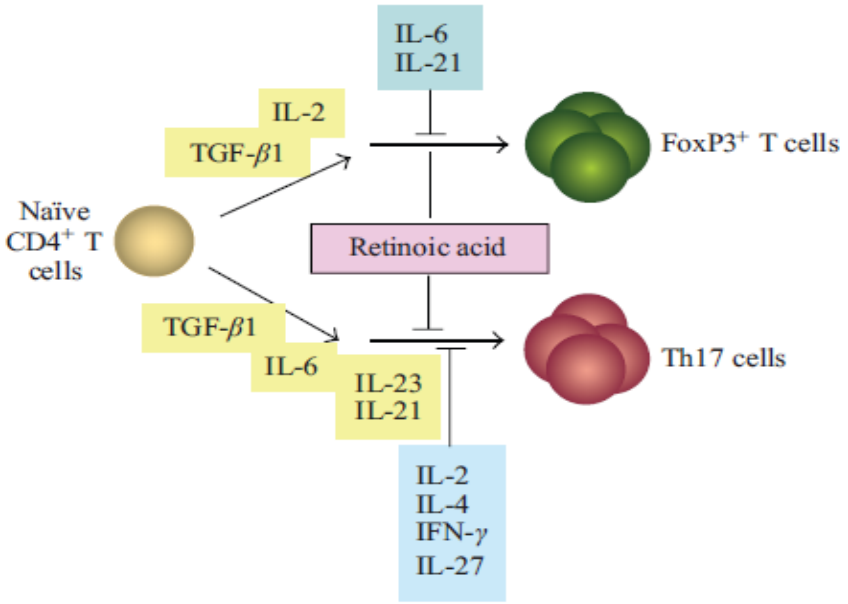

Fig.1.3. A central role for vitamin A in the reciprocal regulation of inflammatory versus regulatory T-cells**.** Within this schema, vitamin A suppresses the polarization of Th17 cells while promoting the induction of T regulatory cells in the context of the noted cytokines.

Several leads point to a possible common stem cell origin for the pancreatic ductal and endocrine cells. Firstly, despite the limited proliferative capacity of islet cells, the formation of new islets from cells associated with the ductal epithelium is achievable. Secondly, cultured mouse pancreatic ductal epithelial cells can produce islet cells. Thirdly, islets generated in vitro, derived from ductal stem cells, can reverse diabetes in diabetic mice [40]. Finally, the insulin-positive cells were found in cytokeratin 19-positive ductal epithelium in both diabetic and non-diabetic cells [41].

The coincidence of pancreatic cancer and diabetes is well known and may reflect the failure of sustaining enough islet mass resulting from the dedifferentiation of ductal cells. Retinoic acid induces transdifferentiation of pancreatic ductal cells into physiologically controlled insulin secreting cells [11, 12, 15, 42].

Based on the above basic research results, we hypothesized that vitamin A working in vivo through retinoic acid can enhance transdifferentiation of the pancreatic duct cells into insulin secreting β-cells that could be protected from death through the immunomodulatory action of vitamin A (retinoic acid) particularly in T1DM human patients.

1.4. SIGNIFICANCE OF THE STUDY

The purpose of this study is to determine whether Vitamin A supplementation improves glycemic control (by stimulating endogenous insulin release) in Type 1 DM.

1.5. HYPOTHESIS

Vitamin A supplementation does not influence glycemic control nor does it improve endogenous insulin secretion.

1.6. OBJECTIVES OF THE STUDY

General Objective:

- To determine whether vitamin A has a therapeutic role in Type 1 Diabetes.

Specific Objectives

- To determine whether Vitamin A supplementation improves glycemic control in patients with Type 1DM
- To determine whether Vitamin A supplementation improves endogenous insulin secretion (rejuvenates β-cell function).
- To determine changes in HbA1c levels following Vitamin A supplementation.
- To determine the impact of Vitamin A on patient's lipid profile.

2. MATERIALS AND METHODS

Study design: This study was an interventional (follow-up) study on 24 Type 1 Diabetic patients diagnosed clinically and attending the Diabetic center at Gondar University Hospital. The study period spanned from beginning of June to end of August 2011.

Study area: Gondar is a historical city with splendid castles from the 17th century, located 750km NW of Addis Ababa and is home for one of the oldest medical schools in Ethiopia: Gondar University Hospital which is a referral teaching Hospital serving an estimated 5 million people in and around Gondar. The Hospital has a chronic illness clinic run by the department of Internal Medicine helping patients with Diabetes (Mondays for T1DM patients and Fridays for T2DM patients taking oral medications), heart disease, hypertension, Asthma. Currently, there are more than 3,000 registered diabetic patients getting diabetes care in this clinic. There are also three satellite health centers at a radius of 35-65km covered by an outreach programme in an attempt to make the service accessible to patients residing in the rural areas.

Study subjects:

Patients attending the chronic illness clinic at Gondar University Hospital who had telephone addresses and registered were chosen and communicated to ensure their participation and those who agreed were enrolled in the study.

Initially around 40 patients were selected and 30 of them agreed to participate. They were randomly assigned by lottery-method in to treatment and control groups where the treatment group received 100,000IU of vitamin A every ten days (three times per month) while controls received only their conventional insulin therapy. One Vitamin A capsule contained 100,000 IU of retinol and 20IU of vitamin E along with it (Vitamin E added for protection of retinol against oxidative damage).

Then both groups of patients were followed for about 6 weeks (a total of 2-3 visits) and during each visit fasting blood sugar was measured. Liver function tests (SGOT and SGPT), C-peptide and HbA1c measured on day one (baseline) and measurements were repeated at the end of the study period i.e. after 6 weeks.

Patients were informed about and followed for Vitamin A toxicity and other serious side effects like hypoglycemia and they were free to contact the investigator for any mishaps, concerns or symptoms related to their diabetes. They were also interviewed to get some clinical data related to their condition like symptoms of liver disease, night blindness, headache, diarrhea (malabsorption), before their enrollment. Body height was measured by the investigator using a stadiometer and their weight taken as well and the information recorded on a data sheet.

Ethical clearance was obtained from the institutional review board of Addis Ababa University which also was accepted by University of Gondar Ethics Committee.

Sample size calculation

The number of participants required in each intervention group, m, is given by:

$$m = \frac{2 \times [Z_{(1-\alpha/2)} + Z_{(1-\beta)}]^2}{\Delta^2}$$

Where z(1- α /2) and z(1- β) represent percentage points of the normal distribution for statistical significance level and power, respectively (see statistical tables), and Δ represents the standardized difference (i.e. the treatment difference divided by its standard deviation).
First calculate Δ, the standardized difference, sometimes called the effect size. In the case of two means, μ 1 and μ2, with a common standard deviation 's', the standardized difference.

$\Delta = \frac{\mu 1 - \mu 2}{S} = \frac{\delta}{s}$ **where δ is the clinically important difference.**

In our case, I expect a mean difference in **glycemic level** of 15mg/dl between the groups with a standard deviation (inter-patient variation) of 10 giving **Δ=1.5**
Hence the calculated sample size for each group**,** Using the values from the table for a significance level of 5%, z (1- α /2) = 1.96, and a power of 95%, z (1- β) = 1.6449, shall be 12. Considering a 25% lost-to-follow-up, a total of 30 patients were recruited.

Inclusion and exclusion criteria

- All adult Patients (>18 years) who were diagnosed clinically with Type 1 Diabetes (insulin-requiring from the outset) will be recruited as study subjects. Type 2 Diabetics and those who were on anti-diabetic oral medications were excluded.
- Patients who gave a verbal and written consent participated.
- Only Patients on "stable regimen" of insulin were included in the study, i.e. those who had frequent dosage adjustments in their recent visits (last 1-3 months) or during the study period were excluded from the study.
- Patients who were already on any form of vitamin supplementation are excluded.
- Patients who developed serious side effects from Vitamin A toxicity during the course of the study were excluded.
- Study subjects who were pregnant or have plans to have pregnancy in the near future were excluded because of Vitamin A's potential teratogenicity.
- Patients who are on medications which could affect blood glucose level like steroids, thyroid hormones, oral contraceptives; thiazide and loop diuretics, phenytoin were also excluded.

- Those patients with diarrhea, severe headaches like migraine, jaundice and significantly elevated levels of transaminases (SGOT and SGPT were also excluded.

2.1. DETERMINATION OF FASTING BLOOD SUGAR

Hexokinase method

The hexokinase method is a reference method for determination of glucose concentration. The method is specific for D-glucose. Under the action of the hexokinase enzyme, D-glucose is phosphorylated with ATP molecule to form glucose-6-phosphate. By the action of glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of NADP, thus formed glucose-6-phosphate transforms to 6-phosphogluconate, whereby NADPH is formed.

The absorbance of NADPH is measured in the UV region (340 or 365 nm). Besides glucose, fructose and mannose can also react in primary reaction. However, Glucose-6-Phosphate Dehydrogenase is specific exclusively for glucose-6-phosphate, thus phosphorylated fructose and mannose do not react in indicator reaction.

A. PRINCIPLE

The enzyme hexokinase (HK) catalyzes the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6phosphate and adenosine diphosphate (ADP). In the presence of NAD, the enzyme glucose-6-phosphate dehydrogenase (G6PDH), oxidizes glucose-6-phosphate to 6-phosphogluconate.The increase in NADH concentration is directly proportional to the glucose concentration and can be measured spectrophotometrically at 340 nm.

1. **Glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP**

2. **G-6-P + NAD $\xrightarrow{G6PDH}$ 6-Phosphogluconate + NADH**

B. Procedure (Automated)

Analysis of fasting blood glucose was done using Hitachi 902 auto analyzer (Roche Diagnostics, Japan) with the following characteristics. A Wavelength of 340 nm at 37^0C And Incubation Time of 180 seconds

2.2. Determination of Hemoglobin A1C

A. Principle:

Total Hb and HbA1c concentrations were determined after hemolysis of the anticoagulated whole blood specimen. Total Hemoglobin (Hb) was measured colorimetrically and HbA1c immunoturbidimetrically. The ratio of both products yields the final percent HbA1c result (%). The anticoagulated whole blood specimen was hemolyzed automatically on COBAS INTEGRA systems (Hitachi 902 Automatic analyzer, Roche diagnostics, Japan) with HbA1c Hemolysis reagent in the predilution cuvette. Erythrocytes were lysed by low osmotic pressure. The released Hb was proteolytically degraded by pepsin, to make the β-N terminal structures more accessible for the immunoassay.

i) **Total Hb** was determined on COBAS INTEGRA systems in the hemolysate using a cyanide free colorimetric method based on the formation of a brownish green chromophore (alkaline hematin D-575) in alkaline detergent solution. The color intensity is proportional to the Hb concentration in the sample at 552nm.The test result was calculated using a fixed factor determined from the primary calibrator chlorhemin.

ii) **HbA1c** was measured on COBAS INTEGRA systems using monoclonal antibodies attached to latex particles. The antibodies bind the β-N terminal fragments of HbA1c.Remaining free antibodies are agglutinated with a synthetic polymer carrying multiple copies of the β-N terminal structure of HbA1c.The change in turbidity is inversely related to the amount of bound glycopeptides and is measured turbidimetrically at **552nm**. A synthetic polypeptide comprising the N-terminal structure of HbA1c is used for calibration.

The reactions are as follows:

1. Total Hb and HbA1c in hemolyzed EDTA blood bind with the same affinity to particles in R1. The amount of binding is proportional to the relative concentration of both substances in the blood.

Glycopeptide + Antibody latex ⟶ Bound Glycopeptides

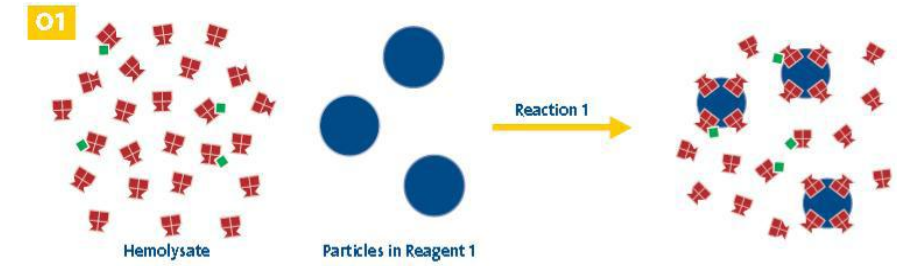

Fig.2.1. Glycopeptide-Antibody latex interaction

2. Mouse anti-human HbA1c monoclonal antibody R2 binds to particle bound HbA1c. Goat anti-mouse IgG polyclonal antibody R3 interacts with the monoclonal mouse anti-human HbA1c antibody and agglutination takes place.

Ab latex + Agglutinator ⟶ Agglutinated Ab latex

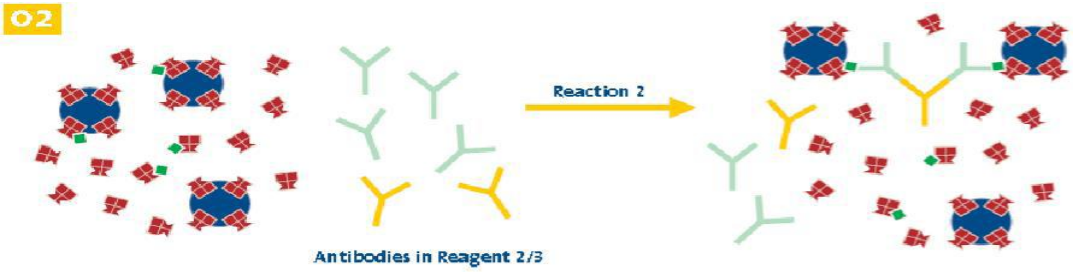

Fig. 2.2. Agglutination reaction

The **absorbance was then measured at 660** nm is proportional to the HbA1c bound to particles, which is in turn proportional to the percentage of HbA1c in the sample.

B. Reagents and Chemicals

1. Haemoglobin Denaturant Reagent(R1)

- Porcine, pepsin
- Buffer pH 2.4
- Preservative

2. Total haemoglobin Reagent (R2)

- Sodium Hydroxide pH 13 0,4%
- Triton 2.5%

3. HbA1C Antibody Reagent

- HbA1C Antibody (mouse) coupled particles, Bovine Serum Albumin
- Buffer pH 8.1, Non ionic surfactant 0,6%
- Proclin 150, 0.1%

4. HbA1C Agglutinator Reagent

- HbA1C hapten covalently attached to the polymer(latex)
- Bovine Serum Albumin, Buffer pH 2,Non ionic surfactant 0.2%,
- Proclin 150, 0.1%

C. Procedures

i. **Specimen collection** and preparation
Venous blood was collected with EDTA from the cubital vein using aseptic technique.

ii. **A hemolysate was Prepared** for each sample:

a) 1 mL Hemolysis Reagent was added into tubes labeled: Control, Patients.
b) Then 20 µL of well mixed whole blood was Placed in to the appropriately labeled reagent tube. It was then mixed.
c) It was allowed to stand until complete lysis was evident.

Determination of total Hb

1. 80µl hemolyzed sample or standard and 1000µl Total Hb reagent (R2) were mixed and incubated for 5 minutes.
2. OD was read at 600nm. This gave the Total Hb.

Determination of HbA1c

1. A sample was added to 500µl latex reagent (Anti-HbA1c monoclonal antibody coated to latex particles)
2. 475µl agglutinator reagent was added to the above mixture and was incubated at 37^0C for 5 minutes.
3. Absorbance was measured at 600nm immediately (A1) and after 2 minutes(A2).

Calculation

The final result was expressed as percent HbA1c/Hb ratio as follows

HbA1c(%)= HbA1c/Hb x 100

2.3. Determination of C-peptide (ELISA)

Background:

Concentration of C-peptide reflects the production of endogenous insulin by the Pancreas. Insulin is first produced as an 86 amino acid residue pre-proinsulin from which a signal peptide is proteolytically cleaved to make proinsulin. Proinsulin is then cleaved into the active hormone, insulin, and an inactive peptide, C-peptide.

Insulin ha an alpha chain with 21 amino acid residues and a beta-chain 31 amino acid residues long connected by disulfide bridge. Insulin and C-peptide are co-secreted and released in equimolar proportions. Insulin has a very short half-life, a fifth of that of C-peptide.

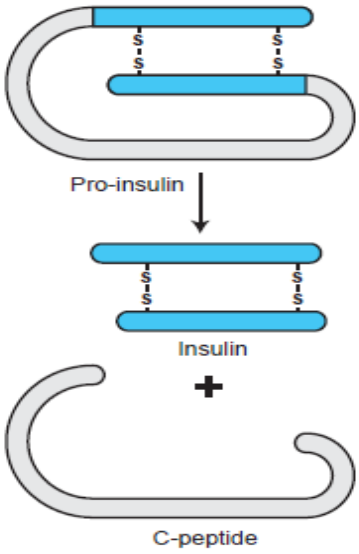

Fig.2.3. C-peptide: a product of insulin production. Proinsulin is cleaved by proconvertases and carboxypeptidases in to insulin and C-peptide; a connecting peptide between alpha and beta chains of insulin which are linked by disulfide-bridge.

A. Principle

The C-Peptide ELISA is a sandwich type immunoassay (CAL BIOTECH, California, and USA). The 96-well microplate is coated with a monoclonal antibody specific for C-peptide. The C-peptide Standards, Diabetes Antigen Controls, and samples are added to the microplate wells with Assay Buffer. After incubation , the wells are washed with Wash Buffer and blotted dry.

The Conjugate (horseradish peroxidase enzyme-labeled monoclonal antibody) is then added, and the microplate is incubated a second time on , washed, and blotted dry. TMB Substrate is added, and the microplate is incubated a third time. Once the third incubation period is complete, Stop Solution is added, and the optical density (OD) is measured by spectrophotometer at 450 nm with a reference wavelength of 620 nm. The intensity of the color generated is directly proportional to the amount of C-peptide in the sample.

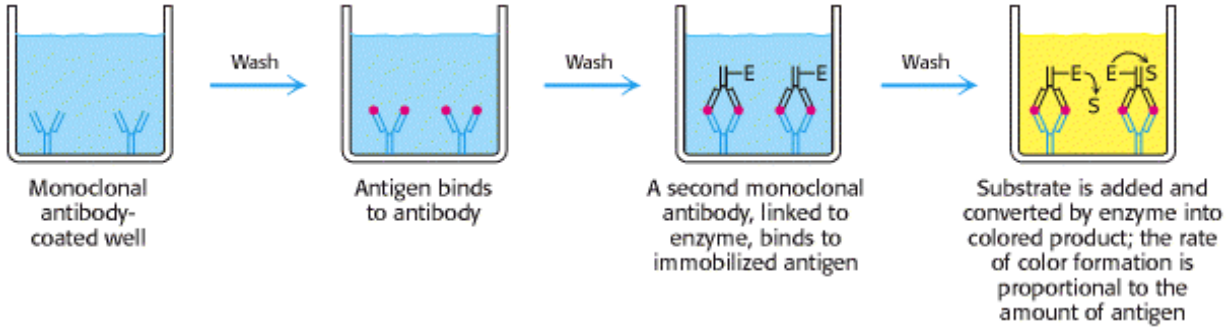

Fig.2.4. Sandwich ELISA, the production of color indicates the quantity of antigen

A. **Reagents &Materials required**

- Precision pipettes with disposable tips capable of dispensing 50 µl,100 µl,1ml
- multi-channel pipette capable of dispensing 50 µl and 100 µl
- Volumetric container and pipettes for reagent preparation
- Distilled water
- Microplate washer or wash bottle.
- Microplate reader with 450nm and 620nm filter

Wash Buffer (21X) was preparedby diluting 25 ml of Wash Buffer (21X) with 475 ml of distilled water.

B. **Procedure**

All reagents were brought to room temperature prior to use. .
Microplate strips were designated for the standards, controls, and number of samples.

1. The microplate wells for each serum sample and standard to be assayed were formatted in duplicate.
2. 50 µl of each reconstituted standard, reconstituted control, and sample were Pipetted into the corresponding wells.
3. Then100 µl of enzyme conjugate was then Pipetted in to each well.
4. The plate was then gently mixed for 15-20 seconds
5. Incubated for 1 hour, at room temperature
6. Liquid was removed from all wells. The wells were washed three times with 300µl of wash buffer.
7. Then 100 µl of TMB Substrate was added into each well.
8. It was incubated for 15 minutes at room temperature.
9. 50 µl of Stop Solution was added into each well and gently mixed for 15-20 seconds on a microplate shaker.
10. The microplate was the placed in a microplate reader capable of reading the absorbance at 450 nm with reference wavelength of 620nm and the absorbance read from each well and the print-out was obtained.

Calculation of results
Manual Calculation:

The Zero Standard was used as a blank with its average value subtracted from each well. The standard concentrations were plotted on the X-axis and the absorbance values on the Y-axis using log/log paper. The sample concentrations were determined by plotting the absorbance of each unknown sample against the standard curve. The corresponding value on the X-axis represents the concentration of the unknown sample.
Conversion factor for Human C-peptide to grams: 1 pmol/L = 3.6 pg/ml

2.4. Determination of Lipid profile

2.4.1. Determination of serum Triglycerides

A. Principle:

The method is based on the enzymatic hydrolysis of triglycerides to glycerol and free fatty acids (FFA) by Lipoprotien Lipase (LPL).The glycerol is phosphorylated by glycerol kinase(GK) to Glycerol-3-phosphate(G-3-P). The later is oxidized by glycerol phosphate oxidase to form Dihydroxy acetone phosphate (DHAP) and H_2O_2. In the presence of Peroxidase and H_2O_2, 4-aminoantipyrine will be coupled with phenol. OD of the chromogen will be measured at 500nm.

Reactions:

Triglycerides + $3H_2O$ —Lipoprotien Lipase→ Glycerol + 3FFA

Glycerol + ATP —Glycerokinase→ Glycerol-3-P + ADP

Glycerol-3-P + O_2 —Glycerophosphate oxidase→ DHAP + H_2O_2

$2H_2O_2$ + 4-amino-antipyrine + phenol —Peroxidase→ Quininoid dye + $4H_2O$

B. Reagents

i) R1 : 150000 U/l Lipoprotien lipase, 800U/l Glycerokinase,4000U/l Glycerol-3-P oxidase,440 U/l Peroxidase, 0.7mmol/l 4 aminoantipyrine,0.3mmol/l ATP and 7.5 mmol/l phenol

ii) R2**:** Glycerol equivalent to a concentration of 200mg/dl(2.28 mmol/l) triglycerides

C. Procedure

To the labeled test tubes, 1.0ml of the working reagent (R1) was mixed with10µl of sample or standard. After 5 minutes of incubation at 37^0C, absorbance was measured at 500nm against the reagent blank.

Calculation:

Triglyceride concentration in the sample was calculated against the absorbance of the standard as follows**:**

Triglycerides (mg/dl) = (A sample/ A standard) x Conc. of the standard

2.4.2. Determination of serum total cholesterol level

A. Principle:

This method involves the use of three enzymes:

Reactions:

Cholesterol Ester —CE→ Cholesterol + FFA

Cholesterol —CHOD→ Cholestenone + H_2O_2

$2H_2O_2$ + 4-amino-antipyrine + phenol —**Peroxidase**→ Quininoid dye + $4H_2O$

B. Reagents

i) R1: 200mmol/l PIPES PH 7.0, 1mmol/l sodium cholate, >250U/l cholesterol esterase, >250U/l cholesterol oxidase, >1KU/l peroxidase, 0.33 mmol/l 4-aminoantipyrine, 4mmol/l phenol, 2g/l non-ionic surfactant, Biocides

ii) R2 : 5.18mmol/l cholesterol standard

C. Procedure

To the labeled test tubes, 1.0ml of the working reagent (R1) was mixed with10µl of sample or standard. After 5 minutes of incubation at 37^0C, absorbance was measured at 546 nm against the reagent blank.

Calculation:

Total cholesterol (mg/dl) = (A sample/ A standard) x Concentration of the standard

Statistical analysis

The statistical software SPSS version 13.0 was used for data entry and analysis. Continuous variables were summarized as means $\pm$S.D. Comparisons among groups of different normally distributed parameters were performed with Student's t test for unpaired data (independent Sample t-test) and paired sample T-test in cases of comparison against baseline values. Significance was set at $P \leq 0.05$. Test of normality was checked by Kolmogorov-Smirnov Test and a test for equality of variance by Levene's test of equality of variance were done for all outcome variables namely, fasting blood sugar, glycated hemoglobin, C-peptide levels, triglyceride and cholesterol levels. Therefore, all the assumptions for independence, normality and equality of variance were fulfilled and hence the parametric independent sample T-test was used for analysis (See Annex C).

2.5. Ethical Considerations:

Ethical clearance was obtained from the Institutional Review Board (IRB) of Addis Ababa University and this was also accepted by the Gondar University Hospital ethics committee.

3. RESULTS

This study was carried out on Type 1 Diabetic patients attending the Gondar University Hospital Diabetic Center. A total of 30 Diabetic patients were recruited randomly assigned to treatment and control groups for the study but for different reasons (mainly lost-to follow-up) only 24 (12 in each group) were followed till the end, out of which 15(62.5%) were males and 9(37.5%) females.

At the time of this assessment, the study subjects who are all Type 1 diabetes mellitus (T1DM) patients ranged in age from 18 to 48 years (mean $\pm$ S.D.,27.54 $\pm$9.1 years). Majority (62.5%) of the patients were aged below 30 years and of these 70.8% were admitted. All but two patients had Diabetic ketoacidosis at first diagnosis (See table 3.1).
Most of the patients were aged < 30 years at onset of diabetes and with insulin treatment from the outset, a classic scenario for type 1 diabetics.

The mean duration of diabetes among controls was 2.2$\pm$1.6years while that of the treatment group was 1.7$\pm$1.1years. Diabetes duration ranges between 3 months and 7years with majority of patients (87.5%) less than three years.

The mean dosage of insulin among treated and control groups are 46.25$\pm$16.4 (IU/day) and 48.63$\pm$10.5 IU/day respectively**.**

As to the type of insulin used, all of them were on lente insulin taking two daily injections subcutaneously and some self-adjustment of their insulin injection is carried out by themselves by about 5IU from their daily dose and sometimes they are forced to reduce their insulin dose when they face shortages of insulin supply. This titration of insulin however didn't happen in this group of patients who participated.

The Body mass index (BMI) is 20.15$\pm$2.18 Kg/m^{2}for treatment group and 19.68$\pm$2.50Kg/m^2 for controls. All of them had a body mass index < 25kg/m^2.

There were no statistically significant differences between control and treatment groups with regard to Age, sex, BMI, duration of disease, dosage of insulin therapy etc**.**

Table 3. 1. Demographic and diabetes related characteristics (N =24) among T1DM patients in Gondar, Ethiopia 2011

Parameter	Control group, N	Treatment group, N	Total N (%)
Age (years)			
< 30	**8**	7	15(62.5)
30-40	**3**	4	7(29.2)
> 40	**1**	1	2(8.3)
Total	**12**	12	**24(100)**
Sex			
Male	8	7	15(62.5)
Female	4	5	9(37.5)
Total	12	12	24(100)
***Admission at Diagnosis(DKA)**			
Yes	10	7	17(70.8)
No	2	5	7(29.2)
****BMI (Kg/m^2)**			
<18			
18-25	4	2	6(25)
	9	9	18(75)

***Fifteen (62.5%) patients were admitted for DKA and two for diabetes education.*

****BMI, body mass index ranges between 16.3 and 24.2Kg/m^2*

Patients had no frequent headaches, night blindness, diarrhea, jaundice and excessive alcohol intake. Except for one patient with retroviral infection who is on antiretroviral therapy, they were not taking any other drugs except their insulin injections twice daily and the vitamin capsules were provided only for the treatment group. This particular patient was on antiretroviral drugs for over four years and it has been two years since she started insulin injections for Type 1 diabetes. All other patients didn't have other diagnosed co-morbidities.

HbA1c is a highly valuable clinical measure of glycemic control particularly with the currently accepted standard of tight or near-normal glycemic control. Though not statistically significant (P=0.165), there was a clinically significant difference in the mean glycated hemoglobin level in the treatment group as compared to that of controls (6.7%versus 8.4%). This is a remarkable improvement in HbA1c level from a clinical standpoint. Supplementation with vitamin A kept it under 7% which is the recommended target glycemic control for type 1 Diabetics. This effect size gives a Cohen's d value of 0.6 which showed beyond medium effect in a practical sense (Annex A).

The pattern of repeated fasting blood glucose measurements correlates well with HbA1c levels ($r = 0.70$, $P<0.01$). Fasting blood sugar level falls by 21% among the treated group which parallels a fall in HbA1c levels.

Table 3. 2. Changes in serum C-peptide level, glycated hemoglobin and fasting blood sugar among Vitamin A treated and control groups. Gondar 2011

	CONTROL GROUP		**TREATED GROUP**	
Parameters	**Baseline**	**After 6 weeks**	**baseline**	**After 6weeks**
Fasting C-peptide levels(ng/dl)	**0.44 ±0.43**	**0.94± 1.92**	**0.48± 0.42**	**1.06 ± 0.96**
****HbA1c (%)**	**7.74±3.14**	**8.44±3.54**	**7.14±2.12**	**6.70±2.28**
Fasting Blood Sugar(mg/dl)	**181± 100**	**156± 92**	**177± 93**	**140 ± 106**

All Values are described as mean ±SD.

****Effect size, i.e. Cohen's d=0.6(medium effect)***

Assessment of β-cell function, as measured by C-peptide levels, is the most suitable primary outcome for pivotal intervention studies and with regard to fasting serum C-peptide levels about 26% of patients were c-peptide positive (>0.6ng/ml) and only 21.4% had a C-peptide>1ng/ml which is considered clinically significant.

The mean HbA1c level among those with negative or low c-peptide levels is markedly elevated and those with higher C-peptide levels usually had lower HbA1c levels.

Besides, patients with C-peptide levels greater than 0.6 ng/ml (0.2 nmol/l) had significantly lower HbA1c levels and were treated with less insulin, indicating better overall glycemic control, as compared with those with levels less than 0.6 ng/ml(See table 3.3). Particularly, HbA1c measurements showed statistically significant difference between the two categories of c-peptide levels (P-value=0.015).

Table 3.3 Comparison of glycemia and insulin requirement as a function of different categories of c-peptide levels at Gondar University Hospital Diabetic center, 2011

c-peptide levels > 0.6ng/ml					
	N	Minimum	Maximum	Mean	Std. Deviation
***HbA1c** (%)	9	4.10	8.30	**5.76**	1.55
Fasting blood sugar (mg/dl)	9	69.00	214.00	**147.76**	44.69
dosage of lente insulin (IU/day)	9	25.00	75.00	**42.22**	14.81
C-peptide levels <0.6ng/ml					
	N	Minimum	Maximum	Mean	Std. Deviation
***HbA1c (%)**	11	3.88	15.80	**9.41**	3.39
Fasting blood sugar (mg/dl)	10	63.70	388.00	**214.66**	116.38
dosage of lente insulin (IU/day)	10	40.00	75.00	**52.50**	10.61

****Mean HbA1c showed statistically significant difference (P-value=0.015)***

There was an increase in triglyceride and total cholesterol levels among the vitamin A treated group, but statistically significant alterations in serum triglycerides levels were not detected in this study. However, the lipid profile for the control group remained almost the same over the study period. (See table 3.4).

Table 3.4. Changes in fasting serum triglyceride and total cholesterol level among treated and control group, Gondar 2011

		Fasting Serum cholesterol levels(mg/dl)		Fasting triglyceride levels(mg/dl)	
		Baseline	**After 6 weeks**	**Baseline**	**After 6 weeks**
TREATED GROUP	**Mean**	**156.00**	***182.45**	**114.18**	**137.00**
	S D	**31.14**	**34.37**	**50.53**	**83.59**
CONTROL GROUP	**Mean**	**157.71**	**153.55**	**119.57**	**121.36**
	SD	**34.145**	**33.32**	**67.45**	**59.18**

Not statistically significant, P >0.05

4. DISCUSSIONS

Most of the patients (87.5%) were aged < 30 years at onset of diabetes and all of them with insulin treatment from the outset, a classic scenario for type 1 diabetics. The mean age of the 24 type 1 diabetic patients was 27.5 $\pm$9.1 years. A recent survey from rural Ethiopia again confirmed the existence of unusually large numbers of young (peak age 25 to 29 years) insulin-treated patients [43].
The underlying cause of type 1 diabetes, loss of β-cell function, has become the therapeutic target for a number of interventions in patients with type 1 diabetes. Even though insulin therapies continue to improve, it remains difficult to achieve normal glycemic control in type 1 diabetes, especially long term. The strongest evidence that the preservation of β-cell function results in improved metabolic control, and consequently fewer end-organ complications, is provided by the DCCT.

Vitamin A, with its immunomodulatory effects, has been ascribed as being relatively deficient in subjects with established type 1diabetes [33] and Vitamin A deficiency is largely considered a problem in the developing world. In other studies, an impairment of vitamin A metabolism has been identified in type I diabetes patients and streptozotocin (STZ)-induced diabetic rats [44,45]. Indeed, recent studies outside the type 1 diabetes arena have noted vitamin A as a major "agent of influence" in the development of what is widely referred to as "oral tolerance" to dietary agents, as well as in the regulation of immune responses,[29] stressing on its immunomodulatory effects.

In the animal models of type 1 diabetes, the NOD mouse and the BB rat, immunomodulatory therapy can alter or block the autoimmune disease process and development of diabetes can be slowed or prevented [28].

HbA1c as a measure of glycemic control should certainly be assessed in any study or trial aimed at showing a beneficial effect on β-cell function. However, given the importance of achieving good glycemic control to reduce complications and because glycemic control strongly influences the decline in β-cell function in type 1 diabetes [46], trials in recently diagnosed type 1 diabetes patients will involve treating all subjects to the same near-normal glycemic target. Consequently, differences in HbA1c between treatment groups will be minimal even when an effective therapy is involved.

To avoid the vascular complications of diabetes, patients are now advised to maintain tight control and keep blood glucose levels as close as possible to the normal range (HbA1c < 7%) by taking multiple daily injections of insulin, frequently monitoring blood glucose levels, and adjusting insulin doses accordingly [47,48].

This study showed the mean HbA1c in the treated group to be 6.7% versus 8.4% for the control group. This remarkable improvement in HbA1c shows a clinically significant benefit of vitamin A supplementation though it failed to show statistical significance (P value=0.165). This is in line with a study from South Korea which suggested possible therapeutic value of Retinoic acid for the prevention of diabetes mellitus progression via inhibition of cytokine-induced β-cell dysfunction [49].

Another study by Van et al. also demonstrated that ATRA treatment promoted in vivo expansion of Treg cells (regulatory T-cells) and induced Treg cell–dependent immune tolerance by suppressing IFN-γ–producing T-cells, without affecting Th17 cells providing novel insights into how ATRA induces immune tolerance in vivo via its effects on T-effector and Treg cells [37].

The improvement in HbA1c level among the treated group was not statistically significant probably because of the low dose of vitamin A used, the short period of supplementation (only 6 weeks) after two weeks of run-in period and the small sample size which under-powered the study. The other reason might be the half-life of HbA1c lasts up to 3 months but the final measurements in this study were taken just after 6 weeks only which is a bit early to pick up a change in HbA1c level even if there was any.

The results from the DCCT study showed that the mean HbA1c for the group that received standard care was 9% where as the mean HbA1c for the group that received special treatment (intensive insulin therapy) to control glycemia was 7%, and the group on intensive insulin therapy exhibited a 76% reduction in retinopathy, 69% reduction of neuropathy, and 44% reduction in nephropathy as compared to the group that received standard care [47].

The current study showed that 25% of patients had HbA1c above 8.6% where as 21% of them HbA1c level exceeding 9.6% showing poor glycemic control. Another Study showed that the risk of overt nephropathy increased substantially when HbA1c was above 9.6% while the risk of severe retinopathy increased already when HbA1c exceeded 8.6 % [50].

The fasting blood glucose measurements correlate well with HbA1c levels as evidenced by a fall in fasting blood glucose among the treated group by 21% which parallels the fall in HbA1c levels in the current study. This correlation of repeated fasting blood glucose measurements with HbA1c levels suggests that the use of HbA1c might be more useful to re-adjust insulin therapy rather than a single measurement of fasting blood glucose on their day of visit for follow-up which is usually every 2-3 months theoretically fitting with hemoglobin's half-life. This notion is supported by another study from the same study area, which noted that fasting blood glucose fluctuates and doesn't show the real picture of glycemic control and hence is unreliable [51].

It is generally accepted that hyperglycemia portends chronic diabetic complications. The benefits expected from "tight" glycemic control are highly salutatory as shown by the Diabetes Control and Complications Trial (DCCT). The rationales for other interventions should be consistent with either enhancing glycemic control per se or adding benefit to an established degree of glycemic control. Since "tight" control achieves less than "pancreatic" control of glycemia, i.e., glycemia at 6 mM rather than 5 mM and glycated hemoglobin at 7% rather than 5%, additional benefits are likely to be achievable.

Type 1 diabetic complications are more directly related to glycemic control than in Type 2 diabetes, as hypertension, lipid abnormalities, obesity and insulin resistance could be involved in the development and/or progression of diabetic complications. Current recommendations regarding glycemic control suggest that HbA1c should be lower than 6.5% in order to substantially delay onset of complications, mainly retinopathy [52].

In the current study, overall mean HbA1c level for both groups at baseline was 7.41 % (range 4% - 15.8%), and the mean fasting blood glucose levels at different points in time during the study (182 mg/dl, 178 mg/dl and 156 mg/dl respectively from baseline to end of study). This obviously is not a good control. In fact, some experts argue about running the risk of hypoglycemia in an attempt to achieve a near-normal glycemic control but it has been shown by the DCCT study that tight glycemic control significantly delays diabetes progression. Besides, hypoglycemic episodes are less common in those with residual β-cell function.

Based on a review of current research evaluating new therapies for type 1 diabetes, the group of international experts convened by the ADA concluded that assessment of β-cell function, as measured by C-peptide levels, is the most suitable primary outcome for pivotal intervention studies of therapies aimed at preservation of β-cell function in patients with type 1 diabetes [53].

In the current study, the mean C-peptide level among the treated group was raised to 1.06ng/ml from a baseline level of 0.48ng/ml (a 55% increase in endogenous insulin secretion) which was statistically significant. This finding is in line with a study by Cobretta et al. which showed that all trans retinoic acid (ATRA) enhanced insulin release from pancreatic β-cells after short-term exposure [30]. In another *in vitro* study by Carbera-Valladares et al., treatment with retinoic acid increased insulin secretion in both fetal and adult islets; the increases on insulin secretion being more pronounced in the mature islets [54].

Studies have linked low levels of C-peptide to diabetes mellitus complications, and evidence suggests that maintaining higher levels of C-peptide is especially beneficial for type 1 diabetics. Type 1 diabetes patients who are able to maintain C-peptide levels have fewer complications and better glycemic control [53].

Thus, in addition to good glycemic control, the preservation of β-cell function is now a therapeutic goal for type 1 diabetes mellitus, and monitoring β-cell function in these patients via C-peptide levels is a rational approach because insulin measurements would be complicated by the presence of exogenous insulin. Besides, rates of severe hypoglycemia and diabetic complications ultimately will be improved by therapies that are effective at preserving β-cell function.

In this study, we tried to investigate the amount of residual insulin release from pancreatic β-cells and the change brought about through Vitamin A supplementation and we found that 26% of patients were c-peptide positive (>0.6ng/ml) and only 21.4% had a C-peptide >1ng/ml. This finding is comparable with the study done by Faber et al. which showed that post–clinical diagnosis, about 15% of individuals still have measurable β-cell function even 5 years later. As can be seen, the percentage in our study is a bit higher (21.4% versus 15%) probably because our patients had shorter mean disease duration of about 2 years only [56-58].

C-peptide levels were also found to be higher among these patients with shorter duration of diabetes probably indicating that Vitamin A supplementation could be more effective if administered early in the course of the disease (primary prevention).

In addition, patients with C-peptide levels greater than 0.6 ng/ml (0.2 nmol/l) had significantly lower HbA1c levels and were treated with less insulin, indicating better overall glycemic control, as compared with those with levels less than 0.6 ng/ml (See table 3.4. above). Particularly, HbA1c showed statistically significant difference between the two c-peptide categories (P-value=0.015). This finding is in line with the results from the DCCT which found that C-peptide levels correlate with overall glycemic control.

Patients with stimulated C-peptide greater than 0.6 ng/ml had significantly lower HbA1c levels and were treated with less insulin, indicating better overall glycemic control, as compared with those with levels less than 0.6 ng/ml. The DCCT also found that there was less retinopathy and nephropathy with preserved β-cell function.

C-peptide levels can also be predictive of autoantibodies. Torn et al. have found that C-peptide levels can predict the presence of autoantibodies (defined as the presence of at least one of the following: islet cell antibodies, GADA, or tyrosine phosphatase antibodies).

A study by Torn et al. determined that a random C-peptide value of below 0.91 ng/ml had a 94% positive predictive value for patients with autoantibodies and a value above 2.42 ng/ml had a 72% positive predictive value for patients without autoantibodies [59].

In this study, 14 out of 17 patients (82.3%) had baseline fasting C-peptide levels below 0.6ng/ml (C-peptide negative) and hence they could have autoantibodies in their serum though direct measurements were not done. Similarly, a recent study from Northern Ethiopia found out that despite the clinical characteristics suggestive of type 1 diabetes, only 61% patients were C-peptide-negative and 35% GADA-positive. Overall, 36% of the total group had immunological or C-peptide characteristics inconsistent with typical type 1 or type 2 diabetes [60].

In the DCCT trial, type 1 diabetic patients with sustained C-peptide secretion showed a significant reduction in the risk for microvascular complications compared with those patients totally lacking C-peptide secretion Stteffes et al. also showed that even modest beta-cell activity was associated with a decrease in the incidence of microvascular complications[61].

In the animal models of type 1 diabetes, the NOD mouse and the BB rat, immunomodulatory therapy can alter or block the autoimmune disease process and development of diabetes can be slowed or prevented [28] and retinoic acid or vitamin A is believed to have immunomodulatory actions. Hence, vitamin A could be beneficial in the treatment as well as prevention of diabetes.

There was a modest increase in triglyceride and total cholesterol levels among the vitamin A treated group, but statistically significant alterations in serum triglycerides levels were not detected in this study, likely due to the low doses of vitamin A used and the short duration of supplementation. Only five patients (21%) had a triglyceride level beyond 150mg/dl before and after treatment.

This finding is consistent with a previous study reporting Retinoid therapy to be associated with hypertriglyceridemia and often with low HDL-cholesterol [62, 63]. Long-term treatment (20 weeks) with etretinate, a vitamn A derivative was reported to improve glucose tolerance in diabetic subjects [64]. However in other studies, controversial data have been reported regarding retinoids' effects on hepatic lipid and lipoprotein metabolism and blood lipid profile. Moreover, the molecular mechanisms underlying retinoid effects on lipid metabolism are complex and remain incompletely understood [65].

None of the patients reported any symptoms of Vitamin A toxicity. This probably is because the dosage of vitamin A used in this study was based on a similar study designed to evaluate the effects of combination of zinc and vitamin A supplementation on biochemical profiles of T1DM who used one-half of a 25,000 IU vitamin A tablet every other day [66]. Besides the tolerable upper intake levels of Vitamin A for adults, defined as the maximal level of daily nutrient intake that poses no risk of adverse effects is 10,000IU/day [67].

There was no significant rise/elevation in SGOT and SGPT levels in both groups both at baseline and at the end of the study period.

4. LIMITATIONS OF THE STUDY

- The immunological diagnosis of type 1 diabetes relies primarily on the detection of auto antibodies in the serum of type 1 diabetes mellitus patients which was not done in the current study. Diagnosis of diabetes was purely clinical.T1DM patients with classic clinical symptoms and requiring insulin therapy for glycemic control from the outset. No immunologic diagnosis.
- Resources were very limited(time and money): We would have recruited more patients, supplemented vitamin A and followed them more frequently and for a longer period at least six months or a year. We could also have determined serum retinol level at baseline and LDL and HDL fractions of the serum lipid profile as well.
- Selection-bias: town patients with telephone-access were included.
- Base-line Vitamin A status of the study subjects was unknown so is their detailed dietary habits, i.e. nutritional intake of pro-vitamins like carotenoids.
- Diabetes classification is quiet tricky in this part of the world for type 1 diabetics have unique presentations as reported from previous studies from this study area.

5. CONCLUSIONS

1. Although the sample size was relatively small, this study showed that Vitamin A improves glycemic control in T1DM patients by enhancing insulin secretion as shown by the slight C-peptide increment. This study has showed clinical benefits of vitamin A supplementation which can't be simply ignored.
2. This study suggested that C-peptide levels correlate well with overall glycemic control and this could potentially mean less retinopathy and nephropathy with preserved β-cell function. Hence, Vitamin A might prevent or delay the onset of diabetic complications.
3. When patients come during their follow up visits we are interested in their objective glycemic level in the preceding month(s) rather than the single FBS measurement on the day-of -visit which obviously doesn't reflect what's been going on. This would certainly affect the quality of care given and would mean a waste of the meager resource at hand.
4. The average age at death of people with Type 1 diabetes of just 32 years was reported from the same area where this study was done obviating the need for a tighter glycemic control as well as finding out and implementing preventive strategies to delay the onset if possible or at least the progression of diabetes. This could potentially have socioeconomic consequences.

6. RECOMMENDATIONS

This study has showed clinical benefits of Vitamin A which can't be ignored. Vitamin A clearly has several important biological actions and more research is needed to fully characterize the effects and roles of long-term Vitamin A supplementation for diabetes mellitus in humans for most of the studies in the past were either animal studies or *in vitro* studies.

Vitamin A may play a role in the treatment of type 1 diabetes; however, to better define the role of vitamin A in the development and progression of type 1 diabetes, high-quality randomized Clinical Trials that measure serum retinol concentration and clinically relevant glycemic outcome measures including c-peptide levels are needed.

Given the high prevalence of vitamin deficiency from previous studies in Ethiopia, this study also indirectly raises the question: is micronutrient deficiency a contributory factor for Diabetes in Ethiopia?

HbA1c measurements are better suited for patient follow-up to ensure a more stringent glycemic control for most of the patients at the chronic illness clinic of Gondar University Hospital have their follow-up visits booked every two to three months and care-providers rely on a single fasting blood sugar measurement on their day of visit with some interview .Off course, a feasibility study shall be done before embarking on it especially from the perspective of cost-effectiveness which was not addressed by this study. The study might have given an indirect clue to the fact that vitamin A could protect or delay the onset of diabetes in high risk groups.

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8. Annexes

Annex A. Cohen's d reflects effect size

Effect size: It estimates the strength of the relationship between two variables (e.g., the correlation coefficient, r; or, when comparing two groups, the standardized magnitude of the **between-group difference**, e.g., Cohen's d). This provides a standard metric for determining whether observed relationships or differences are meaningful in a practical sense:
d= (mean1 – mean2)/ [(SD1 + SD2)/2].
According to Cohen,

d=0.2 is indicative of a small effect (corresponding the 58th percentile);
d=0.5 is a medium effect (69th percentile); and
d=0.8 is a large effect (79thpercentile).

Annex B. Tests for Normality: Kolmogorov-Smirnov Test

		HbA1c (%)	Fasting blood sugar(mg/dl)	cholesterol levels(mg/dl)	triglyceride levels(mg/dl)	C-peptide (ng/dl)
N		22	22	18	18	17
Normal Parameters(a,b)	Mean	7.4141	181.54	156.67	116.28	.465
	Std. Deviation	2.58618	100.27	31.35	55.82	.413
Most Extreme Differences	Absolute	.129	.222	.185	.247	.248
	Positive	.129	.222	.185	.247	.248
	Negative	-.093	-.123	-.128	-.140	-.171
Kolmogorov-Smirnov Z		.603	1.040	.786	1.047	1.024
Asymp. Sig. (2-tailed)		**.860**	**.229**	**.567**	**.223**	**.245**

a Test distribution is Normal.
b Calculated from data

Annex C. Subject information sheet

Title of study: Evaluation of therapeutic benefits of Vitamin A in Type 1 Diabetes

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully (or one of us will read it for you if needed). Talk to others about the study if you wish.
Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. The purpose of the study?

The Purpose of this study is to look in to the benefits of Vitamin A therapy in controlling Diabetes. Some *in vitro* studies showed these benefits but there are only few human studies and none from Ethiopia where micronutrient deficiencies are quite common. This study therefore aims to be an eye-opener in this area. The study aims to reveal if there is any benefit of taking Vitamin A daily in improving management of Diabetes.

2. Potential Benefits of the study

We cannot promise the study will help you but the information we get from this study will help improve the treatment of people with Diabetes and may even give an insight to a possibility of delaying the onset of disease in those people at risk though this is difficult to say from such small scale study. Hence, your participation could mean a lot in opening a new avenue in the future management as well as prevention of Diabetes. Furthermore, we offer this participation as a chance for you to get a close monitoring and a more frequent follow up of your condition as we will have weekly schedules.

3. What is expected of the participant?

You have been invited to participate because you are having the condition to be investigated and a total of about 50 other Diabetics shall be included in the study as well so you are not alone.
If you agree to participant in the study you will be involved in the research for about 2 months. You are expected to:

- Come once every two weeks for a blood test and to meet the researchers for any concerns. There will be a total of 4-6 visits within 2 months.
- You will be taking Vitamin A capsules (100,000IU) once every ten days for a month. The Vitamin A therapy you get is an additional treatment on top of what you are

currently taking so you shouldn't stop or decrease the dose of your current medication and in case you need titration of your current dose report it to the researcher immediately.

- Report immediately if you develop unusual symptoms of hypoglycemia, headache, rash, drowsiness, abdominal pain, nausea and vomiting.

4. Conditions of withdrawal from the study and complaints

Taking part in the research is entirely voluntary. It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive. But your participation could mean a lot in opening a new avenue in the future management as well as prevention of Diabetes.
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions (contact number 0913523935).

5. Possible disadvantages and risks

Vitamin A capsules you will be taking are taken daily as supplements by healthy people worldwide at a lower dose offcourse.It is a well studied drug and the dosage is less than what is taught to cause toxicity so it is safe. However it is not advised to take it while pregnant because of its teratogenicity. So you must **withdraw from the study if you are pregnant or have plans** during the study period.
• In case toxicity arises though chances are remote, the researchers do not have a compensation scheme but would help to alleviate the condition as much as we can. Symptoms of Vitamin A toxicity include headache, rash, drowsiness, irritability, abdominal pain and nausea and vomiting. There could also be symptoms of recurrent hypoglycemia.
• Because of budget constraints we would not be able to cover your travel and other expenses and we offer this participation as a chance for you to get a close monitoring and a more frequent follow up of your condition as we will have weekly schedules.

6. Confidentiality

We will follow ethical and legal practice and all information about you will be handled in confidence. Every information gathered about you and your condition is strictly confidential and to be utilized by the research group members only. In other words, any information carrying your identification shall not be accessible to other people. But the results of this study would be presented and could be published.

Annex D. Consent Form

Title of Project: **Evaluation of therapeutic benefits of Vitamin A in Type1Diabetes Mellitus**

Name of Researcher: Alemseged Woretaw

Please Mark those boxes to show you agreed

1. I confirm that I have read and understand the information sheet dated..... (Version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from Gondar University Hospital, AAU-Medical Faculty, or from regulatory authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature