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Insulin resistance and hyperinsulinemia precede insulin deficiency in hypertransfused patients with thalassemia major

Peter Alexander Merkel
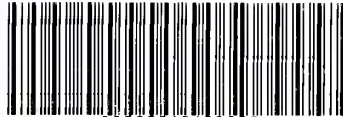*Yale University*

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INSULIN RESISTANCE AND HYPERINSULINEMIA
PRECEDE INSULIN DEFICIENCY
IN HYPERTRANSFUSED PATIENTS WITH THALASSEMIA MAJOR

PETER ALEXANDER MERKEL

1988

Insulin Resistance and Hyperinsulinemia Precede Insulin
Deficiency In Hypertransfused Patients with Thalassemia Major

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirement for the Degree of
Doctor of Medicine

by

Peter Alexander Merkel
1988

ABSTRACT

Insulin Resistance and Hyperinsulinemia Precede Insulin
Deficiency In Hypertransfused Patients with Thalassemia Major

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Diabetes mellitus in patients receiving
hypertransfusion therapy for thalassemia major is usually
attributed to damage to β cells. To examine whether iron
overload might lead to insulin resistance even before the
development of impaired insulin secretion, 12 children with
thalassemia major (4 prepubertal, 8 pubertal) with normal or
only modestly impaired glucose tolerance on chelation
therapy were studied. Each patient received intravenous
insulin (40 mU/m$^2\cdot$min) to raise plasma insulin to $\approx$80 μU/ml
for 2 hours while plasma glucose was maintained at 90 $\pm$ 5
mg/dl by a variable rate glucose infusion (euglycemic
insulin clamp technique). Under these conditions of
euglycemia and hyperinsulinemia, the glucose infusion rate
reflects total body glucose metabolism (M) and is an index
of insulin sensitivity. In prepubertal patients with

thalassemia major, M values (319 ± 23 mg/m^2·min) were similar to those in prepubertal normal controls (315 ± 41 mg/m^2·min, P=not significant). In contrast, insulin sensitivity was markedly reduced in pubertal thalassemics (151 ± 17 vs. 224 ± 15 mg/m^2·min in normal pubertal controls, $p<0.01$). The reduction in insulin sensitivity in patients with thalassemia major was correlated with iron load ($r= -0.77$, $p<0.01$).

The pubertal, but not the prepubertal thalassemics, had an excessive rise in plasma insulin levels after oral glucose ($p<0.001$). Furthermore, in response to a standard 125 mg/dl (6.9 mmol/l) hyperglycemic stimulus (hyperglycemic clamp technique), 2-3 fold greater than normal early and late insulin and C-peptide responses were demonstrated in the pubertal patients.

These data suggest that insulin sensitivity is reduced in children with thalassemia major when they reach puberty due, in part, to chronic iron overload. Compensatory increases in insulin secretion may help maintain normal glucose tolerance in the face of insulin resistance at this stage of the disease. Increased insulin secretion due to insulin resistance may contribute to β-cell exhaustion in thalassemia major.

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NOTE REGARDING PUBLICATIONS AND PRESENTATIONS

Results of this project have been presented at two national meetings:

> The Annual Meeting, American Pediatric Society and The Society for Pediatric Research, Washington D.C., May 1986.

> National Student Research Forum, Galveston, Texas, April 1987. Recipient American Diabetes Association Award for the outstanding paper in diabetes research.

A manuscript describing this project has been accepted for publication:

> Merkel PA, Simonson DC, Amiel SA, Plewe G, Sherwin RS, Pearson HA, Tamborlane WV. Insulin resistance and hyperinsulinemia in patients with thalassemia major treated by hypertransfusion. N Engl J Med (in press).

DEDICATION

This thesis is dedicated to my parents, Eva and Edgar Merkel. Their constant support, encouragement and love has been with me through my many years of study and will always be a tremendous source of strength and inspiration in my life.

CONTENTS

INTRODUCTION

HISTORY OF THALASSEMIA

In 1925, Drs. Thomas B. Cooley and Pearl Lee first reported five cases of children with "... anemia, splenomegaly, and some enlargement of the liver...." They further described a "peculiar" appearance of some of the children "... caused by enlargement of the cranial and facial bones, combined with the skin discoloration."[1] Cooley et. al. realized that while some of the cases were similar to those of von Jaksch's anemia or a leukemia, this new entity deserved separate classification.[2] They suggested that the disease was "... a form of hemolytic anemia, developing in early life and dependent probably on some congenital defect in the hemolytopoietic system."[2]

These early observations and theories proved to be surprisingly accurate and the new syndrome came to be known as Cooley's anemia. This eponym remains in use today but has been largely supplanted by the term "thalassemia" which was first coined by Whipple and Bradford in 1932.[3,4] The term is based on the Greek words "thalassa" meaning "the sea" and "emia" meaning "the blood" reflecting these

authors' observations that most cases occurred in people of Mediterranean descent. In the more than sixty years since Cooley's first description of thalassemia, enormous strides have been made in our understanding of its molecular basis and pathophysiology leading to advances in the diagnosis and treatment of this disease.

MOLECULAR BASIS OF THE THALASSEMIAS

The term thalassemia is used to describe a heterogenous group of inherited anemias characterized by decreased or absent production of one or more of the polypeptide chains which make up human hemoglobin. Most thalassemias involve the polypeptide chains of hemoglobin A1 ($\alpha 2\beta 2$). Thalassemia genes are inherited in an autosomal dominant pattern. When the synthesis of the α-globin molecule is impaired or absent, the resulting diseases are referred to as the α-thalassemias. Similarly, when synthesis of the β-globin molecule is reduced, the resulting syndromes are known as the β-thalassemias. Other, rarer, forms of thalassemia exist which correspond to defects in production of various non-alpha or non-beta hemoglobin chains.[5]

In β-thalassemia, normal β-globin genes are present but are not expressed due to the action of the thalassemia gene complex that alters β-globin gene expression. Although the

basic failure of β-globin chain synthesis most often results
from problems in mRNA template processing, β-thalassemia can
be caused by any of a variety of genetic mutations causing
disturbances at different steps of globin gene expression
and more than 30 different mutations have been described.[5]
The α-thalassemias are caused by similar problems at one or
both of the α-globin loci. Most children with four α-loci
mutations die in utero (fetal hydrops syndrome). Patients
with three α-loci mutations have hemoglobin H disease that
is clinically similar to thalassemia intermedia. Patients
with two or one α-loci mutations are asymptomatic carriers.
Because this investigation concerns β-thalassemia patients
only, further discussion of the α-thalassemias is beyond the
scope of this paper.

β-THALASSEMIAS

Geographic Distribution of β-Thalassemia

The distribution of the gene for β-thalassemia is
centered in the countries bordering the Mediterranean Sea.
Especially high frequencies occur in Italy (up to 5%) and
Greece (5-15%) and children from Italian or Greek American
families comprise by far the majority of patients in this
country.[5] Thalassemia is also found in the Middle East,

Africa and Southern Asia. The gene frequency in black Americans is approximately 0.8%.[6] It is believed that the recent rise in immigration of people of Southeast Asian decent will lead to a sharp increase in the number of new cases of thalassemia major in the United States in the coming decade.[7,8]

The β-thalassemias are usually classified as "minor," "major" and "intermedia" forms based on both genotype and clinical presentation.

Thalassemia Minor

Individuals with thalassemia minor or thalassemia trait are heterozygous for the thalassemia gene and β-globin is synthesized at approximately half the rate of normal, resulting in a mild form of microcytic hypochromic anemia. Laboratory studies reveal reduced mean corpuscular volume, decreased mean corpuscular hemoglobin, decreased hemoglobin (decreased 1.0-2.0 g/dl verses age and sex matched controls) and decreased osmotic fragility.[9] Red blood cell count and reticulocyte counts are normal or somewhat elevated. Peripheral blood smears show some hypochromia, target cells, poikilocytes, ovalocytes, and basophilic stippling. The diagnosis can be made by quantitative hemoglobin electrophoresis showing elevated hemoglobin A_2 levels

(>3.5%). Although most people with thalassemia minor are asymptomatic, some patients complain of easy fatiguability and the anemia may be worsened by pregnancy or iron deficient states.[5] Making a diagnosis of thalassemia trait is useful in explaining abnormal hematological laboratory findings and in excluding iron deficiency anemia with its need for further diagnostic studies and therapy. Establishment of a diagnosis is the rationale for screening ethnic groups and individuals at high risk for carrying the gene in order to provide them with genetic counseling.

Thalassemia Major

In contrast to the relatively benign course of thalassemia minor, the homozygous state for β-thalassemia leads to a severe chronic hemolytic anemia known as thalassemia major. The anemia is so severe that early death occurs unless regular blood transfusion is initiated. The near absence of β-globin production in these patients leads to low or absent levels of hemoglobin A_1. Although there is an increase in gamma and delta-chain synthesis, the response does not compensate for the loss of beta-chain production. Since the rate of α-globin production remains normal, the total number of α-chains greatly outnumber non-α chains. These "unbalanced" α-chains aggregate within red blood cells

into unstable inclusions, or "Fessas bodies."[5,10,11] These
inclusion bodies cause death of early red blood cells in the
bone marrow and markedly shortened survival of those that
reach the circulation. Along with the total decrease in
hemoglobin content, this process results in production of
hypochromic, microcytic and poikilocytic red blood cells.

Thalassemia Intermedia

Approximately 10% of patients with homozygous β-
thalassemia have clinical disease with degrees of anemia and
symptoms that are less severe or delayed than those
described below. This syndrome is known as thalassemia
intermedia. These patients vary greatly in their clinical
course. They may require only minimal transfusion support
early in the disease, but splenectomy and full transfusion
therapy may eventually be needed.[5]

CLINICAL FINDINGS IN UNTREATED THALASSEMIA MAJOR

The clinical manifestations of untreated thalassemia
major are a direct result of hemolytic anemia and become
apparent after the infant is six months old, when fetal
hemoglobin synthesis normally diminishes. Presenting

clinical features can include pallor, jaundice, irritability, growth retardation, and abdominal swelling due to liver and spleen enlargement.[5] Cardiac dilation due to the severe anemia may be observed. Compensatory marrow hypertrophy occurs causing the dramatic skeletal changes of thalassemia which Cooley first described: frontal bossing, maxillary enlargement with dental abnormalities, severe osteoporosis and early epiphyseal fusion.[2,5] The liver, kidneys and spleen all suffer enlargement due to extramedullary hematopoiesis. Splenomegaly also results from the organ's role in red blood cell removal. Without transfusion therapy, the life expectancy of patients averages less than four years.

Laboratory findings in untreated thalassemia major are those of a severe uncomplicated hemolytic anemia. After about six months of age the hemoglobin level falls as low as 3.0-4.0 g/dl. Peripheral blood smear reveals the classic hypochromic, microcytic cells with poikilocytes, target cells as well as cells with the Fessa bodies described above. The reticulocyte count is between 2-8%. The red blood cells are resistant to hemolysis, a finding Cooley first noted and one that helps distinguish the disease from other forms of hereditary hemolytic anemia such as congenital spherocytosis.[2] The bilirubin is elevated to levels of 2.0-4.0 mg/dl. Hemoglobin F is the predominant hemoglobin fraction (20-60%), yet the total hemoglobin level

is still very low. The ratio of hemoglobin $A_2:A_1$ is increased from the normal 1:30 to less than 1:20.[5]

THERAPY FOR THALASSEMIA MAJOR

Prevention: Genetic Counseling

The best treatment for thalassemia major is, of course, prevention. This can only be attained by preventing the births of infants homozygous for thalassemia. To achieve this goal, large-scale programs for genetic screening, prenatal diagnosis and genetic counseling have been established for populations at high risk for carrying the thalassemia gene. Screening for thalassemia trait can be accomplished by measuring mean corpuscular volume and follow-up quantitative hemoglobin A_2 determination in patients with microcytosis.[9,12] Prenatal diagnosis of thalassemia major can be made by fetal blood sampling and globin chain incorporation studies[13,14] or by DNA polymorphisms in cells recovered by amniocentesis[15] or chorionic villus biopsy.[16,17] Decisions by at-risk families not to have children as well as therapeutic abortions by women carrying affected fetuses are options some families involved in the screening programs have taken. Implementation of comprehensive education and screening

programs for high-risk populations in the 1970's resulted in a significant decrease in the number of new cases of β-thalassemia major through the early 1980's and has been spectacularly successful in Cyprus where more than 90% of the births of affected fetuses have been prevented.[8,18] Continued effort in such preventive measures must be maintained and modified to account for any population shifts of at-risk groups.

Hypotransfusion Therapy

The untreated patient with thalassemia major has a very short life expectancy. It was recognized early that transfusions were necessary to sustain the life of these patients. Because of concern over the development of iron overload, transfusions were given sparingly. Therefore, the childrens' hemoglobin was sometimes 5.0-6.0 g/dl or lower and they were often symptomatic. The cardiomegaly, erythroid hyperplasia and severe skeletal deformities that occur in untreated patients were still present in the minimally transfused patients. Furthermore, even with this transfusion schedule, a tremendous iron burden developed over the years causing multiple organ disturbances including cardiac failure, growth and endocrine abnormalities and liver dysfunction. The life expectancy of patients with

thalassemia major on this transfusion schedule averaged 15-20 years.[5]

Hypertransfusion Therapy

The approach to treating patients with thalassemia major changed dramatically in 1964 when Wolman published the results of a retrospective cross-sectional survey of thalassemic patients comparing their average hemoglobin levels with their clinical conditions during transfusion therapy.[19] He showed that compared to patients whose hemoglobin levels were maintained between 4.0-5.9 g/dl, those patients with hemoglobin levels maintained between 8.0-9.9 g/dl had significantly better short-term clinical outcomes. Patients with higher hemoglobin levels displayed less growth retardation, fewer skeletal abnormalities, including facial disfigurement, less degrees of hepatosplenomegaly and less severe cardiomegaly. After this report, many centers began using transfusion regimens that kept hemoglobin levels above 10.0 g/dl. This protocol came to be known as "hypertransfusion" therapy. Numerous studies have demonstrated the distinct advantages of hypertransfusion therapy: normalization of growth, suppression of erythropoiesis, decrease in liver, spleen, and cardiac enlargement. Most importantly, by eliminating

the anemia and marrow hyperplasia, this regimen allows patients to lead full, active lives without the physical disfigurement previously seen.[5]

Hypertransfusion therapy has become the standard regimen for treating patients with thalassemia major in the United States and other countries economically capable of its implementation. One center's recent recommendations for a "supertransfusion" protocol is the maintenance of baseline hemoglobin levels above 11.5 g/dl, a level felt to be a compromise between the goal of maintaining a normal hemoglobin (>14.0 g/dl) and the increased transfusion requirement that would be required to achieve such a level. Such hemoglobin levels can be achieved by transfusions of 10 ml/kg of filtered washed packed red blood cells given every three weeks.[20] The Yale-New Haven Hospital Thalassemia Clinic maintains hemoglobin above 10.0 g/dl by transfusion of 15 ml/kg of washed packed red blood cells every 4-5 weeks.

Although the greater number of transfusions leads to an increase in the amount of iron received by the patient, this may be offset somewhat by a decrease in intestinal iron absorption due to correction of anemia.[19] Nevertheless, iron overload remains the major cause of morbidity and mortality in thalassemic patients. Thus, adjuvant forms of therapy are designed to increase red blood cell survival and increase iron excretion.

Splenectomy

Before the adoption of transfusion therapy, patients with thalassemia major often required early splenectomy due to massive splenomegaly which caused mechanical symptoms, thrombocytopenia and neutropenia.[5] With current hypertransfusion regimens, massive splenomegaly does not occur, although some organ enlargement is inevitable.[20] However, splenectomy is still indicated when there is evidence of a shortened survival time of transfused red blood cells. After splenectomy, transfusion requirements decrease and, therefore, the patient's acquired iron burden is reduced. The operation is usually performed when the patient is at least six years old to allow for the development of humoral immunity to bacteria, especially encapsulated organisms, such as Streptococcus pneumoniae and Haemophilus influenzae.[5,20]

Experimental and Future Therapies

In 1980 Propper et. al. reported on the use of neocytes, red blood cells with a mean age of 20-30 days compared with the mean age of 60 days for regularly transfused red blood cells. Such neocytes are harvested from donors using a continuing flow cell separator similar

to that used to collect white blood cells.[21] It was shown
that transfused neocytes have prolonged in vivo survival
compared to traditionally transfused red blood cells. Other
studies, however, have not shown significantly decreased
blood requirements with neocytes (Bove JR and Pearson HA,
1988, personal communication). Furthermore, this therapy
involves extreme cost and exposure of patients to an
increased number of blood donors. These uncertainties have
rendered neocyte therapy still experimental.[20]

Bone marrow transplantation is theoretically a cure for
thalassemia major but has carried significant risks. The
first successful bone marrow transplant of a patient with
thalassemia major was accomplished in 1982.[22] This was
followed by a number of trials in both infants and older
children. A recent series by Lucarelli et. al. in 40
patients 10-18 years of age reported a 70% success rate but
a 25% mortality rate.[23] The therapy currently requires
histocompatible sibling marrow donors. While a potentially
promising therapy, bone marrow transplantation for
thalassemia major remains experimental and awaits further
progress in risk-reduction. The increased use of the
procedure for genetic and oncologic indications should
continue to help improve the technique's effectiveness and
safety.

The modification of marrow erythroid differentiation
through the use of chemotherapeutic agents such as 5-

azacytidine and hydroxyurea is another experimental approach being considered for patients with thalassemia major.[24-26]

IRON OVERLOAD IN HYPERTRANSFUSED PATIENTS WITH THALASSEMIA MAJOR

Iron Balance

The normal homeostasis of iron, a crucial element for hemoglobin synthesis as well as many other intracellular mechanisms, is maintained through complex interactions of plasma proteins, the reticulo-endothelial system and tissue and red blood cell stores. Dietary iron is absorbed in the upper small intestine. In the circulation iron combines with apotransferrin to form transferrin, a β-globulin molecule that acts as a carrier for iron in the plasma. Approximately 70% of the body's iron pool is normally present in heme compounds (hemoglobin and myoglobin). Another 15-30% is stored in the liver as ferritin, formed when iron combines with apoferritin, and a smaller amount of iron is present as hemosiderin, made up of large aggregates of ferritin. When senescent red blood cells are destroyed within the reticulo-endothelial system, the free iron is transported by transferrin for use in either new hemoglobin synthesis or storage in tissues.[27]

The human body has flexibility in its capacity to maintain iron balance by self-regulating iron absorption. If tissue iron accumulates due to increased oral intake, the storage capacities of apoferritin and transferrin, both usually only partly utilized, become saturated and the rate of gastro-intestinal iron absorption decreases sharply. Mucosal iron deposition itself can interfere with iron absorption. On the other hand, total body iron deficiency causes increased binding capacity of transferrin and decreased iron storage pools resulting in increased gastro-intestinal absorption.[27] However, the body's ability to excrete iron is severely limited.

Transfusions are not the only sources of iron in thalassemic patients. During the hypotransfusion era, thalassemic patients were calculated to have hemosiderosis and total body iron burdens that exceeded the amount of iron known to have been acquired through transfusions. It was postulated that increased gastro-intestinal iron absorption accounted for this difference.[28] Early work with iron tracer studies demonstrated that patients with hemoglobinopathies and other forms of refractory anemias absorb dietary iron far in excess of normal non-anemic individuals.[29] Erlandson et. al. in 1962 demonstrated that patients with thalassemia major had an increase in intestinal iron absorption compared to normal children when the patients' hemoglobin levels were 6.0-8.5 g/dl but had

normal intestinal iron absorption when their hemoglobin levels were maintained at 9.5-12.6 g/dl.[30] Furthermore, this study found no increased rate of intestinal iron absorption in patients with thalassemia trait. They proposed that the increased iron absorption is somehow related to the increased ineffective erythropoiesis seen when patients' hemoglobin levels are low. Iron absorption decreases markedly when hemoglobin levels are restored to normal. Thus, the increased iron administered during hypertransfusion therapy may be offset somewhat by decreased gastro-intestinal iron absorption due to prevention of severe anemia.

Patients with thalassemia major receive approximately 5-15 mg of transfused iron per day (based on 200 mg of iron per 250 ml of packed red blood cells) and will be treated with hundreds of units of blood in their lifetime.[31,32] These patients are incapable of excreting significant amounts of this excess iron and, if left untreated, inevitably develop severe hemosiderosis. By 11 years of age, the average hypertransfused patient has received approximately 28 grams of iron with 1-2 grams being the body's normal iron stores.[32] Increasing age and, therefore, increasing number of transfusions results in a state of iron overload.

Clinical and Pathological Complications

The adoption of hypertransfusion therapy has greatly improved the quality and duration of life for patients with thalassemia major. However, a complication of this therapy, transfusional hemosiderosis, is now the major cause of morbidity and mortality in these patients.

Iron overload was first discussed by Whipple and Bradford in 1936.[3] Studies have demonstrated iron deposition in multiple organs with pathological findings similar to those seen in idiopathic hemochromatosis.[28,33] Schafer et. al. also found similar clinical manifestations of cardiac, endocrine and hepatic damage in non-thalassemic adult patients given intensive short-term transfusion support.[34] The most common pathologic and clinical findings of acquired iron overload are described below.

Cardiac Complications

Cardiac damage and arrhythmias have long been recognized as the most severe complications of acquired hemochromatosis in thalassemic patients and remain today the chief cause of death in most patients. In 1964 Engle reported on the cardiac status of a series of patients with thalassemia major or thalassemia intermedia who had been

receiving traditional hypotransfusion therapy.[35] In that series, 80% of the patients had cardiac involvement including pericarditis, atrial and ventricular arrhythmias and congestive heart failure. She noted that the cardiac complications generally became evident in the second decade of life. Congestive heart failure resulted in the death of 23 of 24 patients with this diagnosis. Engle ascribed these clinical events to hemochromatosis which was pronounced in all patients autopsied. Ehlers et. al. in 1980 reported on a second group of patients at the same institution finding very similar results.[36] The patients in this study who died from congestive heart failure were 9-33 years old with a mean age at death of 18. The patients in both of the above studies were not treated with iron chelation therapy. In 1979 Leon et. al. studied a group of transfusion dependent anemia patients with radionuclide cineangiography and demonstrated a significant decrease in ejection fraction in the transfused patients compared to normal controls.[37] Furthermore, the magnitude of left ventricular dysfunction was inversely correlated with the number of transfusions. Similar results were found in patients with thalassemia major studied by Freeman et. al. in 1983.[38] The results of chelation therapy on cardiac function are discussed below.

Hepatic Damage

In 1954, Ellis demonstrated liver fibrosis and siderosis at autopsy in 11 of 13 patients treated conventionally for thalassemia major.[28] He described pathological changes very similar to those seen in idiopathic hemochromatosis. Other studies reported similar pathological findings in thalassemia major[33] and transfusion-acquired iron overload.[34] Liver function tests in patients with thalassemia major sometimes reveal hyper- or hypogammaglobulinemia, moderate decreases in coagulation factor levels, and elevated transaminase levels.[5,34] Some of these changes may be secondary to transfusion-acquired viral hepatitis which may lead to chronic liver disease in thalassemic patients.[39] Iron deposition can be carcinogenic and one case of hepatocellular carcinoma has been reported in a patient with thalassemia major.[40] Infection with hepatitis B virus may further predispose patients to liver malignancy. As patients with thalassemia survive longer, liver malignancies may become more common and careful screening may be needed.

Endocrinologic Changes

Multiple endocrinopathies have been associated with
thalassemia major and the role of iron overload in these
findings is not always clear. Before the adoption of
hypertransfusion therapy, patients with thalassemia major
were regularly found to have growth retardation thought
secondary to their anemia.[5] Hypertransfusion protocols have
resulted in improved growth. However, in a study of 250
patients with thalassemia major begun on chelation therapy,
37% showed growth that was 2 standard deviations below age-
and sex-matched controls.[41] It must be noted that many of
the patients in this study were not begun on deferoxamine
therapy until later in life. Therefore, the results on
growth of early aggressive chelation is not yet known. The
delay or absence of sexual maturation in patients with
thalassemia major is very common and is felt to be due to
hemosiderotic damage of the hypothalamus.[42-45]

Iron deposition has been demonstrated in virtually all
endocrine glands studied including thyroid, parathyroid,
adrenal, testes and pancreas.[33,42,44,45] Furthermore,
studies have found deficiencies in pituitary, parathyroid,
adrenal, testicular and pancreatic function.[42,44-49]
Problems with sexual maturation are believed to be secondary
to hypothalamic-pituitary dysfunction and/or possible
gonadal hormonal insensitivity resulting in the

hypogonadotropic hypogonadism seen in thalassemia
major.[44,45,50] Hypoparathyroidism has been reported in
patients with thalassemia major and demonstrated by
provocative stimulation tests.[44,48,51,52] The
hypoparathyroidism seen in thalassemia major is felt
secondary to acquired iron overload. Some authors have
reported elevated thyroid stimulating hormone levels in
patients with thalassemia major.[47,48] However, most studies
indicate that despite iron deposition in the thyroid gland
and some thyroid function test abnormalities, patients with
thalassemia major are generally euthyroid.[42,43,47,48,53,54]

Iron Chelation Therapy: Deferoxamine

Without an effective means of removing excess body iron
stores, hypertransfused patients with thalassemia major
rapidly develop severe hemosiderosis with its accompanying
cardiac and endocrinologic complications. Phlebotomy
therapy, while effective in some iron overloaded conditions,
is clearly out of the question in such transfusion-dependent
anemic patients. Therefore, an iron chelating pharmacologic
agent was long sought after. Various agents were
investigated including penicillamine, EDTA, and tricalcium
diethylene triamine pentacetate but all were found
unsatisfactory.[5]

In 1960, deferoxamine (also referred to as desferrioxamine), a sideraphore obtained from <u>Streptomyces pilosus</u> was demonstrated to be a highly specific iron chelator.[5,55] Oral deferoxamine is not well absorbed. The medication was initially given by intramuscular injection, a route of administration that proved to be very painful. Studies in the late 1970's demonstrated that deferoxamine-induced iron excretion could be greatly increased by slow, continuous intravenous administration.[5,56] Subsequently, Graziano et. al. demonstrated that continuous subcutaneous administration of deferoxamine was nearly (79%) as effective as the intravenous route of administration.[57] Subcutaneous administration can now be accomplished at home through the use of portable infusion pumps and this is now the method of choice for deferoxamine therapy. Some treatment protocols combine intravenous deferoxamine administration during transfusions with up to 7 days per week subcutaneous infusion schedules.

In 1968 Wapnick et. al. reported that ascorbic acid deficiency was frequently seen in people with iron overload.[58] Further investigation demonstrated that oral doses of ascorbic acid repleted body stores and significantly increased deferoxamine-induced urinary iron excretion in subjects with transfusion acquired hemosiderosis.[59] Subsequent studies have confirmed these results and ascorbic acid replacement therapy in moderate

(100 mg/day), non-cardiotoxic doses is now a regular
component of iron-chelation therapy.[5]

Hypertransfused patients with thalassemia major treated
with a regimen of chronic, slow subcutaneous deferoxamine
and ascorbic acid repletion have been able to excrete up to
100 mg of iron in urine per day.[5] This response may
diminish over time despite the fact that serum ferritin
levels and calculated iron burden remain increased in these
patients.[20,60,61]

Deferoxamine therapy is generally well tolerated but
the potential toxicity of chronic use is a concern. Some
patients suffer irritation at the infusion sites but
infection, tachyphylaxis or hypersensitivity are only rarely
seen.[5] Sensorineural hearing loss, retinal toxicity, visual
impairment and possible cataract formation have been
reported with high dose deferoxamine therapy.[62-64] These
impairments were reversible in only a few of the patients
with discontinuation of deferoxamine. Regular auditory and
ophthalmologic exams have been suggested for all patients
receiving deferoxamine.

Compliance with using the subcutaneous infusion
protocol has also proven to be a problem, especially among
adolescent patients.[5,20,65,66] Despite the inconvenience,
the great expense and the possible toxicity of therapy,
deferoxamine is the only known effective iron chelation
agent available and will remain the mainstay of therapy in

hypertransfused patients with thalassemia major until a better agent is available.

Effectiveness of Deferoxamine

Since its introduction, investigators have studied deferoxamine's ability to affect the clinical course of patients with thalassemia major. In 1974 Barry et. al. reported the results of a randomized prospective trial of intramuscular deferoxamine chelation therapy in 18 hypertransfused patients with thalassemia major followed with serial liver biopsies.[60] After five years, non-chelated control patients had increased liver iron concentrations compared to the chelation-treated patients who showed minimal changes in liver iron stores. Furthermore, hepatic fibrosis increased in the control group but remained stable in the chelated patients. The two groups showed no significant differences in clinical progression. However, pubertal development was delayed in 4 of 5 untreated patients but in only 1 of 4 chelated patients. It is likely that clinical differences were not seen because the study only covered five years and the patients were not yet in their twenties, when the more severe complications of the hemosiderosis become apparent. In a small uncontrolled trial, Cohen et. al. demonstrated

that liver iron stores could be depleted and the progression of liver fibrosis stopped through even more intensive deferoxamine therapy.[67]

Because chronic subcutaneous deferoxamine therapy is relatively new, no large series of patients have been followed from infancy to their twenties to examine the clinical benefits of maximum chelation therapy. Nevertheless, some studies have shown improved cardiac function or at least delayed onset of ventricular failure with chelation therapy.[38,66,68-72] Kaye and Owen in 1978 reported on 28 patients with thalassemia major ages 7-23 years.[68] They found that of 11 patients considered inadequately chelated, 7 developed "major" cardiac arrhythmias while none of the 17 "well-chelated" patients developed arrhythmias. Freeman et. al. in 1983 demonstrated that early subclinical left ventricular dysfunction correlated with increased iron burden and could be reversed in some patients after intensive chelation therapy.[38] In 1985 Wolfe et. al. examined cardiac status in patients with thalassemia major started on iron-chelation therapy after age 10.[69] They found that while 12 of 19 "non-compliant" patients were affected by cardiac disease, only 1 of 17 compliant patients was. Giardina et. al. in 1985 reported on patients with thalassemia major begun on iron-chelation therapy and followed for five years.[70] Their results, while only short term and preliminary, indicated that improved

cardiac health could be attained and progressive myocardial dysfunction delayed with chelation therapy. The best results were seen in the patients who had yet to reach their twenties, the age of greatest cardiac risk. Grisaru et. al. in 1986 reported improved left ventricular function in 20 patients compliant with chelation therapy and deterioration of ventricular function in 15 non-compliant patients.[71] A study of the population involved in the current investigation also revealed preliminary findings of reduced cardiac damage in patients compliant with chelation therapy.[66]

If cardiac disease, the leading cause of death in thalassemia major, could be prevented with chelation therapy, then life expectancy should rise. Modell et. al. in 1982 reported the first evidence that aggressive deferoxamine therapy results in increased life expectancy compared to untreated patients.[73] However, their conclusions were based on data collected from a group of patients 20 years of age or younger. While future randomized trials of treated verses untreated patients would be unethical, continued follow-up of patients treated since infancy will allow for more definitive conclusions. There is reason to be optimistic that deferoxamine therapy, if started at an early age, will markedly improve the outcome of patients with thalassemia major.

CARBOHYDRATE TOLERANCE IN THALASSEMIA MAJOR

Pancreatic Iron Deposition

Most autopsy studies of patients with thalassemia major reveal severe hemosiderotic pancreatic involvement. Diffuse pancreatic fibrosis and iron deposition in both acinar and islet cells have been described.[28,33] Both of these studies compare the pancreatic findings in thalassemia major to idiopathic hemochromatosis.

Previous Studies of Diabetes Mellitus in Thalassemia Major

Diabetes mellitus is a well known complication of thalassemia major that generally affects patients beyond their teenage years. However, the pathophysiology of these patients' carbohydrate intolerance is not totally clear. Diabetes mellitus in thalassemia has usually been attributed to insulin deficiency from the toxic effects of iron deposited in pancreatic islets.[28,33,46,47,74-79] An experimental model of acquired hemochromatosis was developed in 1979 by Awai et. al..[77] These investigators reported that rats treated with a regimen of intraperitoneal injection of iron, as ferric nitrilotriacetic acid,

developed diabetic symptoms of hyperglycemia, glycosuria and ketonuria. These rats showed decreased insulin responses to oral glucose loading compared to normal controls. Histopathology of the iron-treated rats showed marked iron deposits in the liver and pancreas though only small amounts of iron were detected in the β cells. When the iron-treated rats were phlebotomized, their diabetic symptoms disappeared and their pancreases showed no evidence of iron damage and a normal number of β-cell granules was present. These authors felt that their results indicated the cause of diabetes in hemochromatosis was either a disturbance in iron metabolism or iron deposition in pancreatic islet cells.

While isolated cases of diabetes mellitus in thalassemia were known,[28,80,81] and Engle[35] reported 3 cases in 1964, Lassman et. al. in 1974 were the first to systematically study and describe a group of patients with thalassemia major with documented diabetes mellitus.[46,47] They reported that of 8 patients treated with lifelong conventional transfusion therapy, 3 had impaired carbohydrate tolerance and 2 had insulin dependent diabetes mellitus. They also measured insulin levels during the oral glucose tolerance tests and found 30-minute insulin levels in the thalassemics to be significantly below their controls. The authors concluded that these results were consistent with the theory that diabetes mellitus in thalassemia major was a result on iron deposition in the pancreas.

Subsequent series of patients with thalassemia major reported similar findings of hyperglycemic, hypoinsulinemic responses to oral glucose tolerance tests. In 1976, McIntosh studied 9 patients with thalassemia major finding 7 patients with chemical diabetes mellitus, 4 of which had high glucose and low insulin responses to glucose loading.[75] He, too, felt the carbohydrate intolerance was secondary to deficient insulin secretion due to pancreatic iron deposition. Saudek et. al. in 1977 studied 26 thalassemic patients with oral glucose tolerance tests with 13 patients displaying abnormal glucose tolerance of which 4 were considered "diabetic."[76] Zuppinger et. al. in 1979 found 19 of 22 patients with thalassemia major exhibiting abnormal glucose tolerance with affected patients being hyperglycemic and hypoinsulinemic during the test.[78] Kattamis et. al. studied thalassemics younger than 13 years with intravenous glucose tolerance tests finding 6 of 27 patients with abnormal results and the thalassemics as a group having increased glucose and decreased insulin responses to the intravenous glucose.[82]

In contrast to the above studies, some investigators have reported increased rather than reduced insulin responses to oral glucose in some hypertransfused patients with thalassemia major. Flynn et. al. in 1976 reported the results of 40 oral glucose tolerance tests performed on 19 patients with thalassemia major over a number of years.[48]

They found 5 of 19 patients to have symptomatic diabetes mellitus and low insulin levels. However, 3 of the 5 patients with diabetes mellitus had earlier glucose tolerance studies which had showed increased insulin levels in the face of only modestly impaired carbohydrate tolerance. Finally, some of the 13 patients with normal glucose tolerance had increased insulin responses to the glucose load. These authors suggested that insulin resistance followed by eventual islet-cell failure was a possible explanation for the carbohydrate intolerance observed in some of the patients.

Other studies support these findings of hyperinsulinemia and proposed an etiology for the insulin resistance. In 1977 Costin et. al. reported on 10 conventionally transfused patients with thalassemia major studied before, and for 5 years following, the institution of hypertransfusion therapy.[49] At the onset of the study, only 1 of the 10 patients displayed abnormal glucose tolerance on oral glucose tolerance testing. However, after 4 years of hypertransfusion treatment, half of the 8 surviving patients had abnormal oral glucose tolerance tests. The mean peak insulin levels of the 4 patients with normal glucose tolerance were significantly higher than the control subjects' as wells as the patients' own pretreatment levels. The authors concluded that insulin resistance was an important factor explaining the progressive development

of carbohydrate intolerance and that increased insulin levels were required to maintain normoglycemia. They proposed that the etiology of this insulin resistance was liver cirrhosis secondary to hemosiderosis. They also, however, acknowledged that progressive β-cell damage was an additional factor. Similarly, Dandona et. al. in 1983 studied 15 transfusion-treated patients (14 with thalassemia major and 1 with red blood cell aplasia) who had biopsy-proven hemosiderotic hepatic damage.[83] Fourteen of the 15 patients had normal glucose tolerance tests but had significantly increased insulin responses during the test compared to controls. One patient had overt diabetes mellitus. These investigators thought that the hyperinsulinemia might represent a compensatory response induced by insulin resistance secondary to transfusion-acquired hemosiderotic hepatic damage. Interpretation of these findings is limited, however, because plasma glucose are very variable during oral glucose tolerance testing. Furthermore, augmented late insulin secretion due to loss of early insulin release, as seen in type II diabetics[84-86] could explain the elevated glucose and insulin levels observed in these patients.

THE CURRENT INVESTIGATION

In the present study, the euglycemic insulin clamp, hyperglycemic clamp, and oral glucose tolerance test were employed to determine whether thalassemia major or its treatment with frequent transfusions alters insulin sensitivity, insulin secretion, or both before the development of diabetes mellitus. The study was designed to avoid the problems of bias in regard to age and disease state of subjects that were present in previous investigations in this field. The findings of the present study indicate that increasing iron burden is accompanied by impaired insulin sensitivity in thalassemic patients and that increasing insulin secretion appears to preserve near normal glucose tolerance at this stage of the disease.

METHODS

SUBJECTS

Patients with thalassemia were drawn from the
Thalassemia Clinic of the Yale-New Haven Hospital Medical
Center. The clinic serves patients with thalassemia major
and thalassemia intermedia. Almost all the patients are of
Greek or Italian ancestry. Eligibility criteria included:
age $\geq$ 8 years; treatment with frequent transfusions (15 ml
packed erythrocytes per kg body weight every 4-6 weeks to
maintain a hemoglobin level above 9.0-10.0 g/dl) and
deferoxamine (1.5-2.0 g per kg per day, up to 6 days per
week) given by continuous subcutaneous infusion over 8-10
hours; absence of diabetes[87]; and the ability to give
informed consent. Fourteen patients who met these criteria
were invited and 12 agreed to participate.

Clinical data are summarized in Table 1. There were 4
prepubertal (Tanner I) subjects aged 10.2 ± 0.5 years (mean
± standard deviation) and 8 pubertal (Tanner II-V) subjects
aged 16.0 ± 2.8 years. Three of the pubertal patients
(numbers 7, 9 and 11) carry the diagnoses of thalassemia
intermedia. However, they all were requiring similar

transfusion and chelation treatments as the 9 thalassemia major patients and for purposes of this study were considered as patients with thalassemia major. All subjects were in good general health, were normally active, were taking no medications other than deferoxamine and ascorbic acid, and were free of overt cardiac disease. Several had mild elevation in serum aspartate aminotransferase (SGOT) concentration (Table 1) but serum direct bilirubin levels were normal. No patient had detectable antibodies to human immunodeficiency virus or hepatitis B virus, unless hepatitis B vaccine had been given. Subjects less than 18 years of age showed no evidence of growth retardation (attained heights between the 5-75 percentiles for age) and the weight of each patient was within the mean ± 1 standard deviation of the expected weight for their height,[88] except in one modestly overweight subject (patient number 6). All had elevated serum ferritin levels (Table 1). Chelation therapy was assessed to be good in 10 patients and fair in 2 (Table 1). Compliance with chelation therapy was estimated by frequency of deferoxamine infusions per week: good ≥ 5, fair 3 or 4, or poor <3 infusions per week. They had received between 155-585 units of packed red blood cells during their lifetime. Anti-islet cell antibodies were assayed in the thalassemic patients (except for 2 prepubertal patients) in the laboratory of Dr. George Eisenbarth (Joslin Research Laboratory, Boston, MA). None of the patients had a positive titer.

Forty-four healthy children (11 prepubertal and 33 pubertal) served as age-matched controls (Table 1). All had normal fasting glucose and insulin levels, had no recent illnesses, and were taking no medications. Preadolescent and adolescent control subjects were of similar age, height, weight and body surface area as corresponding preadolescent and adolescent thalassemic subjects (Table 1). Subjects and their parents gave written informed consent before taking part in the study and the protocol was approved by the Human Investigation Committee of the Yale University School of Medicine.

PROCEDURES

All subjects were admitted to the Children's Clinical Research Center for 3-4 days for performance of euglycemic or hyperglycemic clamp studies and glucose tolerance testing as well as annual evaluations of cardiac and hematologic status. Admissions were timed to coincide with the patients' next scheduled blood transfusion which was generally given after completion of the studies. Developmental staging[89] was assessed by a physical examination on the day of admission. The subjects remained relaxed and were entertained throughout the studies by movies.

All studies were performed in the morning following a
10-12 hour overnight fast. Two intravenous catheters were
inserted prior to the clamp studies: one in an antecubital
vein for administration of test substances and the other in
a hand or distal forearm vein of the contralateral arm for
blood sampling. The blood sampling hand was placed in a
heated box (60-65 °C) to "arterialize" blood samples.[90]
After a rest period of at least 30 minutes, baseline fasting
blood samples were obtained for measurement of plasma
glucose and insulin. The euglycemic and hyperglycemic clamp
techniques have been described in detail elsewhere[91] and
will only be briefly outlined below.

Euglycemic Insulin Clamp

The euglycemic insulin clamp procedure was developed in
order to estimate tissue sensitivity to insulin in vivo.
With this technique, insulin is administered as a primed
continuous infusion for 2 hours, in a dose (adjusted for
height and weight) designed to raise plasma insulin
concentrations to the same level in all subjects. Because
the plasma glucose is "clamped" at the same level and the
increment in insulin is similar in all subjects, the amount
of glucose required to maintain euglycemia provides an index
of insulin sensitivity. When insulin is infused in the dose

used in the present study (40 milliunits per square meter of
body surface area per minute), endogenous glucose production
is usually totally suppressed.[92,93] Therefore, the amount
of infused glucose reflects the amount of glucose being
metabolized by the tissues of the body.

For the euglycemic clamp protocol, a prime-continuous
infusion (40 mU/m^2·min) of regular human insulin (Squibb-
Novo, Princeton, NJ) was administered for 120 minutes. In
each subject, the plasma glucose concentration was "clamped"
at approximately 90 mg/dl (5 mmol·l) by simultaneous
infusion of a 20 percent glucose solution. The glucose
infusion rate was adjusted to maintain euglycemia on the
basis of plasma glucose determinations made at the bedside
every 5 minutes. Blood was also obtained at 20 minute
intervals for insulin measurements.

Results in thalassemic patients were compared with data
from 19 healthy controls: 6 Tanner I, prepubertal subjects
(age 10.1 ± 1.0 year, mean ± standard deviation) and 13
Tanner II-V, pubertal subjects (age 15.3 ± 3.7 years) who
were studied under the same conditions. Results in some of
these normal controls have been reported elsewhere.[94]

Hyperglycemic Clamp

The hyperglycemic clamp procedure provides a means to more precisely quantitate insulin secretion in human subjects. With this procedure, exogenous glucose is given intravenously to raise plasma glucose levels by the same amount in each subject and plasma insulin responses to this standardized stimulus are determined. By rapidly raising plasma glucose levels toward the hyperglycemic goal within 20 minutes, the early (first phase) insulin response can be quantified accurately and independently.[91]

Hyperglycemic clamp studies were performed in the Tanner II-V thalassemic subjects during a subsequent visit at least 1 month after the euglycemic insulin clamp study. With this procedure, the plasma glucose level was acutely raised by 125 mg/dl (6.9 mmol/l) above fasting values over a 5-15 minute period according to a standardized algorithm.[91] Plasma glucose was maintained at this hyperglycemic plateau for the remainder of the study (120 minutes) by determination of the glucose concentration at 5 minute intervals and appropriate adjustment of a variable rate 20% glucose infusion. Blood samples were also obtained for measurement of plasma insulin and C-peptide at 0, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, and 120 minutes.

Results in these patients were compared with those from 9 healthy Tanner II-V controls (age 15.7 ± 3.0 years) who were studied under the same conditions.

Oral Glucose Tolerance Test

Oral glucose tolerance tests were also performed in the morning following an overnight fast, two days before or after the euglycemic clamp procedure. Glucose was ingested at a dose of 1.75 g per kg body weight to a maximum of 100 g and blood samples were obtained at -15, 0, 30, 60, 90 and 120 minutes for measurement of plasma glucose and insulin.

Results in the thalassemic subjects were compared to those in 5 Tanner I, prepubertal (age 9.7 ± 2.2 years) and 11 Tanner II-V, pubertal (age 14.8 ± 2.0 years), normal subjects.

BIOCHEMICAL MEASUREMENTS

Plasma glucose was measured by the glucose oxidase method using a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). Plasma insulin[95] and C-peptide (Diagnostic Products Corporation, Los Angeles, CA) were measured by double antibody radioimmunoassay. Plasma ferritin was measured by a solid support radioimmunometric assay procedure (Ramco, Inc., Houston, Texas).

CALCULATIONS AND ANALYSIS

Under steady-state conditions of euglycemia during the insulin clamp study, the rate of glucose infusion provides and index of insulin-stimulated glucose metabolism. This rate (expressed as milligrams per square meter of body surface area per minute) was calculated during 20-minute intervals and adjusted for deviations from the target plasma glucose level of 90 mg/dl (5 mmol/l). This "space correction" corrects for the addition or removal of glucose from the body's glucose space.[91] The average glucose infusion rate required during the last 60 minutes of each clamp study was used for comparison.

During the hyperglycemic clamp, the rate of glucose metabolism (expressed as milligrams per square meter of body surface area per minute) was calculated during 20-minute intervals and adjusted for deviations from the target plasma glucose level of 125 mg/dl above fasting. This adjustment is the same "space correction" as calculated for the euglycemic clamp. In addition, the hyperglycemic clamp results require a correction for urinary glucose loss. The plasma insulin and C-peptide responses during the hyperglycemic clamp study are biphasic; first (0-10 minutes) and second phase (10-120 minutes) responses were calculated as the mean hormone concentration during the respective time periods.[91]

The General Clinical Research Center CLINFO system was used for data collection, collation and analysis. Demographic data are presented as means ± standard deviation and all other data are expressed as means ± standard error. Two group comparisons were made by two-tailed Student's unpaired t-tests and comparisons between 3 or more groups by analysis of variance. Correlations between variables were examined by least squares linear regression analysis.

RESULTS

EUGLYCEMIC CLAMP

In prepubertal thalassemic children, the rate of glucose infusion required to maintain plasma glucose at approximately 90 mg/dl (319 ± 23 mg/m^2·min) was similar to values in normal prepubertal control children (314 ± 41 mg/m^2·min, p=not significant), Figure 1. As previously reported,[94] insulin-stimulated glucose metabolism was reduced in normal pubertal control subjects (to 224 ± 15 mg/m^2·min, p<0.01 vs. prepubertal controls). However, this puberty-associated decline was significantly more pronounced in thalassemic subjects to 155 ± 18 mg/m^2·min, p<0.01 vs. pubertal thalassemics (Figure 1). Among thalassemic subjects, responses to insulin were inversely correlated with the number of transfusions received (r= -0.77, p<0.01; Figure 2), but not with serum ferritin levels (r= -0.26, p= not significant; Figure 3).

ORAL GLUCOSE TOLERANCE TEST

Plasma glucose and insulin responses to oral glucose are shown in Figures 4 and 5. The glucose and insulin responses were within the normal range in prepubertal thalassemic subjects. In contrast, carbohydrate tolerance was modestly impaired in pubertal thalassemic patients, in spite of marked hyperinsulinemia. As shown in Figure 5, the area under the insulin response curve was markedly greater in pubertal thalassemics than pubertal controls (15.3 ± 1.7 vs. 6.3 ± 0.6 mU·min/ml, $p<0.001$ vs. other three groups).

In thalassemic subjects, the area under the insulin response curve was inversely correlated with the rates of insulin-stimulated glucose metabolism determined with the euglycemic clamp procedure ($r=-0.85$, $p=0.002$; Figure 6).

HYPERGLYCEMIC CLAMP

First and second phase plasma insulin and C-peptide responses to a standard 125 mg/dl (6.9 mmol/l) increment in plasma glucose in pubertal thalassemic and pubertal controls are compared in Figure 7. Both first and second phase insulin responses were markedly higher in pubertal thalassemic subjects compared to pubertal controls (phase one: 79 ± 14 μU/ml or 569 ± 101 pmol/l vs. 26 ± 2 μU/ml or

187 ± 14 pmol/l, p<0.01; phase two: 115 ± 26 ± 2 μU/ml or
828 ± 187 pmol.l vs. 47 ± 3 U/ml or 338 ± 22 pmol/l p<0.01).
Similarly, the first and second phase rise in circulating
C-peptide concentrations were significantly greater in
pubertal thalassemics than in controls (p<0.01, Figure 7).
Despite higher insulin concentrations in the thalassemics,
the rate of exogenous glucose infusion required to maintain
plasma glucose at 125 mg/dl (6.9 mmol/l) above fasting was
not significantly different between the pubertal thalassemic
subjects (372 ± 46 mg/m^2·min) and the pubertal controls
(398 ± 57 mg/m^2·min, p=not significant).

PATIENTS WITH DIABETES

Also presented are the results of sequential glucose
tolerance tests in two female thalassemic patients who were
excluded from the study because of the presence of diabetes.
Their glucose intolerance was diagnosed from oral glucose
tolerance testing. Neither patient had yet begun any
dietary or pharmacologic therapy at the time of these
studies. As shown in Figure 8, both patients showed
enhanced insulin responses to oral glucose in their teenaged
years when glucose tolerance profiles were observed in
association with reduced insulin responses.

DISCUSSION

Diabetes mellitus is a common, late complication in β-thalassemic patients requiring frequent transfusions; a problem that has not been totally prevented by use of the iron chelating agent deferoxamine.[96] Development of diabetes in this setting has generally been thought to be due to a direct, toxic effect of iron on the pancreatic islets leading to insulin deficiency.[28,33,46,47,75,77-79] However, a surprising degree of variability in insulin responses to oral glucose has been observed in hypertransfused thalassemic patients; low, normal and even high insulin levels have been reported.[34,46,48,49,75,76,83,97]

Several factors help explain the widely varying insulin responses to oral glucose loading that have been observed in thalassemic patients. Most studies were cross-sectional surveys of entire clinic populations that included patients of different ages, sexual maturity and durations of treatment which could impact on insulin secretion. These studies failed to stratify patients with normal glucose tolerance, impaired glucose tolerance and frank diabetes. Such differences in carbohydrate tolerance produce marked differences in the glycemic stimulus to the β cell.

Insulin resistance has been suggested as a cause of carbohydrate intolerance in thalassemia major.[48,49,83] However, the issue of insulin sensitivity in thalassemia major has never been directly studied but only inferred from results of oral glucose tolerance tests that showed exaggerated insulin responses. The oral glucose tolerance test is inadequate for assessing the potential contribution of insulin resistance which itself could affect insulin secretion in these patients.[91] Furthermore, differences in insulin responses to oral glucose could be related to time-dependent changes in insulin secretion that occur during the course of the disease. To circumvent these problems, the euglycemic and hyperglycemic clamp techniques were used to quantify insulin action and insulin secretion under controlled conditions in patients with thalassemia and in healthy age-matched control subjects. To focus on early changes in carbohydrate metabolism in thalassemia, only young subjects without diabetes were included in the study.

Insulin resistance is defined as a diminished biological response to endogenous or exogenous insulin. Such decreased response may be due to insufficient or ineffective hormone-receptor interactions or defective post-receptor processing. Either process would result in a decrease in the biological response to insulin.[98]

Thalassemia and its treatment with multiple transfusions could alter carbohydrate metabolism by

impairing insulin's action on target tissues. Such insulin resistance has been observed in a number of physiological and disease states. The most common causes are obesity and type II diabetes mellitus. These insulin resistant states are often accompanied by hyperinsulinemia with either normoglycemia or hyperglycemia. Hyperglycemia itself may induce insulin resistance. If overt diabetes mellitus develops in such patients, very large doses of insulin are often required to attain adequate control. Other causes of insulin resistance include acanthosis nigricans, growth hormone excess, glucocorticoid excess and lipotrophic diabetes.[98,99]

Use of the euglycemic and hyperglycemic clamp techniques have enabled investigators to examine insulin sensitivity in a variety of clinical settings. With these methods, decreased insulin sensitivity has been demonstrated in types I and II diabetes mellitus in both obese and lean patients.[92,93,100] Amiel and colleagues at Yale adapted the euglycemic insulin clamp technique for studies in children.[94] With this procedure, they demonstrated that puberty is associated with insulin resistance in both normal and diabetic adolescents. Furthermore, the decrease in insulin-stimulated glucose metabolism seen in puberty was not seen in adult subjects who demonstrated the same insulin sensitivity as prepubertal subjects. This decline in insulin sensitivity in puberty was also demonstrated by

Block et. al. in 1987 in a very similar study of healthy children using the euglycemic insulin clamp technique.[101] Normal control data generated by the Amiel study also provided the opportunity to determine whether insulin responsiveness might be altered in hypertransfused thalassemia patients.

The data from the current investigation suggest that there is no inherent defect in glucose metabolism in thalassemia, since normal insulin secretion, glucose tolerance, and peripheral insulin sensitivity were observed in prepubertal thalassemic children. These findings are consistent with previous studies which failed to find a genetic link between thalassemia major and diabetes mellitus.[46,102,103] Pollack et. al. studied 85 patients with thalassemia major and thalassemia intermedia for HLA-A, B, C and DR antigens.[103] They found no significant association between thalassemia and HLA-linked factors known to be related to the development of diabetes mellitus. This was true for both the group as a whole and the 8 patients with clinical diabetes. The absence of anti-islet cell antibodies in any of the thalassemic subjects (even in the older patients with modestly impaired carbohydrate tolerance) is in agreement with a previous study of islet-cell antibodies in 8 thalassemia major patients aged 15-22 with chemical or insulin-dependent diabetes[104] and along with the HLA studies suggests that an underlying autoimmune process is unlikely.[105-107]

Nondiabetic pubertal thalassemic patients, on the other hand, were found to be markedly insulin resistant, even when the normal decline in insulin sensitivity that occurs with puberty[94,101] is taken into account. During the euglycemic clamp studies, insulin-stimulated glucose metabolism was reduced by over 30% in the pubertal patients when compared with age-matched controls. This impairment in insulin-stimulated glucose metabolism was also evident during the hyperglycemic clamp procedure. In those studies, total glucose disposal was similar in pubertal thalassemics and controls, despite 2-3 fold higher circulating insulin concentrations in the thalassemics. Insulin resistance was correlated with an increased number of transfusions suggesting that this acquired defect may have resulted from chronic iron overload in muscle and liver tissue.

In view of the age of the subjects, radioactive tracer techniques were unavailable for use to determine whether the reduction in insulin action in the pubertal thalassemics was occurring at the level of the liver, muscle or both. However, tracer studies in normal and insulin resistant adult subjects show virtually complete suppression of hepatic glucose production during 40 $mU/m^2 \cdot min$ insulin infusions.[92,93] Therefore, it is reasonable to assume that peripheral tissues, rather than the liver, account for the insulin resistance that was observed. Increased iron content in muscle tissue, as reported in chronic iron

overload[108] might be particularly important, since muscle has been shown to be the principle site of glucose utilization under both euglycemic and hyperglycemic conditions[100,109]. The cellular mechanisms leading to this resistance, receptor versus post-receptor abnormalities, remain to be established.

Serum ferritin levels did not correlate with the degree of insulin resistance in the thalassemia major patients in the present study. This finding is somewhat surprising since changes in serum ferritin levels have been thought to reflect total body iron stores in iron-overloaded patients[110-112]. In studies of multiply-transfused patients, the number of lifetime units of blood transfused was closely correlated with both serum ferritin and liver iron concentration only in patients who had received 50-100 units of blood.[112,113] At higher levels of transfusions the increase in ferritin was much less dramatic. Moreover, Saudek et. al. reported no correlation between serum ferritin levels and patients' ages, transfusion history, or degree of glucose tolerance.[76] Interestingly, as in the current investigation, they noted significantly increased age and number of transfusions in the patients with abnormal glucose tolerance compared to those with normal glucose tolerance. This relationship was most striking in the subset of patients with definite diabetes mellitus. Inflammation and liver disease[113,114] both raise a subject's

serum ferritin level. Differences in the measurement technique for total ferritin,[115,116] also interfere with this value's usefulness as a measure of body iron stores. Thus, it is possible that transfusion history may be a better indicator of total iron burden and degree of hemosiderotic damage than serum ferritin.

Insulin resistance in the pubertal thalassemic patients was accompanied by exaggerated increases in plasma insulin concentrations during oral glucose tolerance testing, particularly early in study. Although this finding could represent a compensatory increase in insulin secretion to overcome insulin resistance, such a cause and effect relationship can not be definitely established with such data in as much as plasma glucose levels were higher in the thalassemic patients. To evaluate this issue further, hyperglycemic clamp studies were performed. This procedure provides an identical hyperglycemic stimulus to all subjects and allows estimation of both first and second phase insulin responses.[91,117] It is particularly noteworthy that both first and second phase insulin responses were increased the pubertal thalassemic subjects. This observation stands in contrast to the characteristic loss of first phase insulin release that occurs early in the course of type II diabetes[84-86] and suggests that the evolution of carbohydrate intolerance in these two disease processes is not strictly comparable.

Hyperinsulinemia in patients with thalassemia might result from either increased pancreatic insulin release or decreased hepatic clearance of insulin, due to liver disease caused by iron overload. Insulin is synthesized and secreted in the β cell as a prohormone consisting of the insulin molecule and the C-peptide fragment. When secreted, both insulin and C-peptide are released in equimolar amounts. C-peptide levels were, therefore, measured during the hyperglycemic clamp to provide a more reliable index of changes in insulin secretion because hepatic extraction of this proinsulin fragment is negligible.[118] The observation that both first and second phase C-peptide responses were enhanced in the pubertal thalassemics supports the view that oversecretion of insulin rather than impaired insulin degradation was primarily responsible for the hyperinsulinemia observed in these patients. This conclusion is supported by recent autopsy findings that β-cell mass is increased in iron overloaded patients with normal glucose tolerance.[119]

On the basis of the findings of this investigation, a mechanism may be proposed to explain the changing nature of carbohydrate metabolism in thalassemia. In the early stages of the disease thalassemic patients show no carbohydrate intolerance or altered insulin sensitivity. However, with increased iron burden, peripheral sensitivity to insulin decreases even in the face of chelation therapy. At this

stage, compensatory increases in insulin secretion serve to maintain near normal carbohydrate homeostasis, implying that impairment of insulin action precedes defects in insulin secretion in thalassemia. Ultimately, insulin secretion fails to keep pace and overt diabetes develops, as illustrated by the glucose tolerance test results in the two patients with diabetes. Thus, the appearance of diabetes in thalassemic patients may be due to a combination of insulin resistance and insulin deficiency; the deficiency due to either exhaustion of β cells[120] or iron deposition in islet cells or a combination of these factors.

Current chelation therapy has not prevented the development of carbohydrate intolerance and diabetes mellitus in many hypertransfused patients with thalassemia major. Further studies are needed to determine the biochemical basis for the insulin resistance in thalassemia. Such investigation might also provide valuable insight into the pathophysiology of insulin resistance seen in other conditions such as type II diabetes. Additionally, it will be important to determine whether adjunctive therapy with sulfonylurea hypoglycemic, that have been shown to increase insulin sensitivity,[121,122] might delay or prevent the development of insulin-dependent diabetes in hypertransfused thalassemic patients by reversing insulin resistance and insulin oversecretion.

TABLE 1: CLINICAL CHARACTERISTICS OF STUDY SUBJECTS

Patient #	Sex	Tanner Stage	Age (years)	Height (cm)	Weight (kg)	Surface Area(m²)	Ferritin (ng/ml)	Hemoglobin (g/dl)	Transfusions (total units)	SGOT (U/L)
1	M	1	9.7	130	28	1.0	2800	10.0	182	37
2	F	1	9.8	125	28	1.0	1850	8.9	155	69
3	M	1	10.6	142	33	1.1	1480	9.8	229	26
4	M	1	10.7	145	34	1.2	2050	9.2	198	38
5	F	2	12.4	145	38	1.2	883	9.8	272	26
6	M	2	13.1	148	52	1.5	4940	10.8	343	57
7	F	3	13.5	158	58	1.6	3900	8.2	263	122
8	M	3	14.9	160	64	1.7	3370	12.1	450	130
9	F	4	17.1	162	55	1.6	3350	9.6	400	88
10	F	4	18.6	152	54	1.5	1470	8.5	585	74
11	F	5	19.1	150	47	1.4	2950	9.3	360	39
12	F	5	19.1	153	54	1.5	2500	10.7	545	47

Tanner I (mean ± SD)   10.2±0.5 136±10 31±3 1.1±0.1
Tanner II-V (mean ± SD) 16.0±2.8 153±6   53±8 1.5±0.2

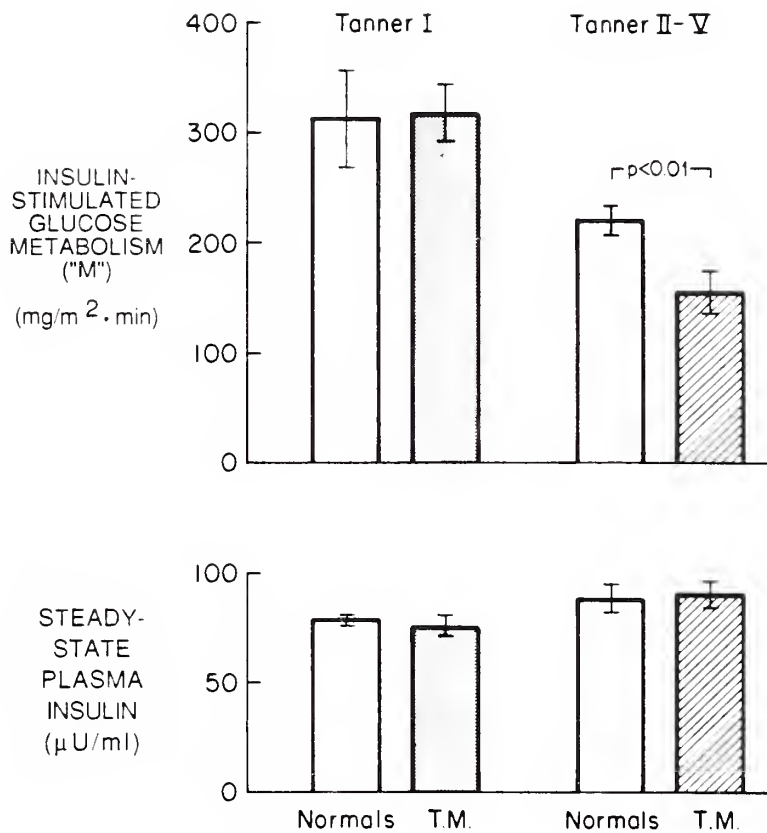| Normal Controls: (mean ± SD)	N	Sex	Age (years)	Height (cm)	Weight (kg)	Surface Area(m²)
Tanner I	11	8M/3F	9.9±1.5	137±8	30±5	1.1±0.1
Tanner II-V	33	16M/17F	15.3±2.9	162±11	55±11	1.6±0.2

Figure 1: Rates of insulin-stimulated glucose metabolism (upper panel) and steady-state plasma insulin concentrations (lower panel) during the euglycemic insulin clamp in prepubertal (Tanner I) and pubertal (Tanner II-V) subjects. Values (means ± standard error) in prepubertal thalassemics (TM) are indicated by the stippled bars, pubertal thalassemics by the hatched bars and aged-matched normal controls by the open bars. P value refers to the significance of difference between pubertal thalassemics and pubertal controls.
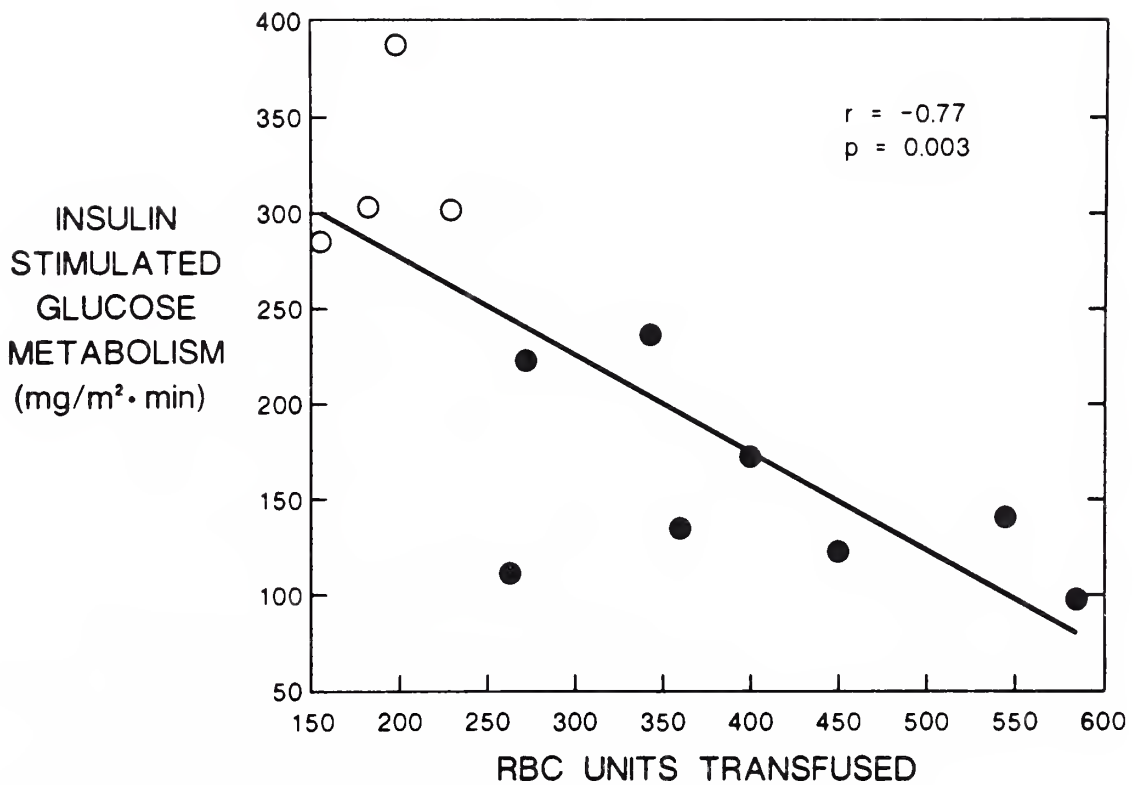
Figure 2: Relationship between insulin-stimulated glucose metabolism, as determined by the euglycemic insulin clamp procedure, and the number of transfusions received in patients with thalassemia major.
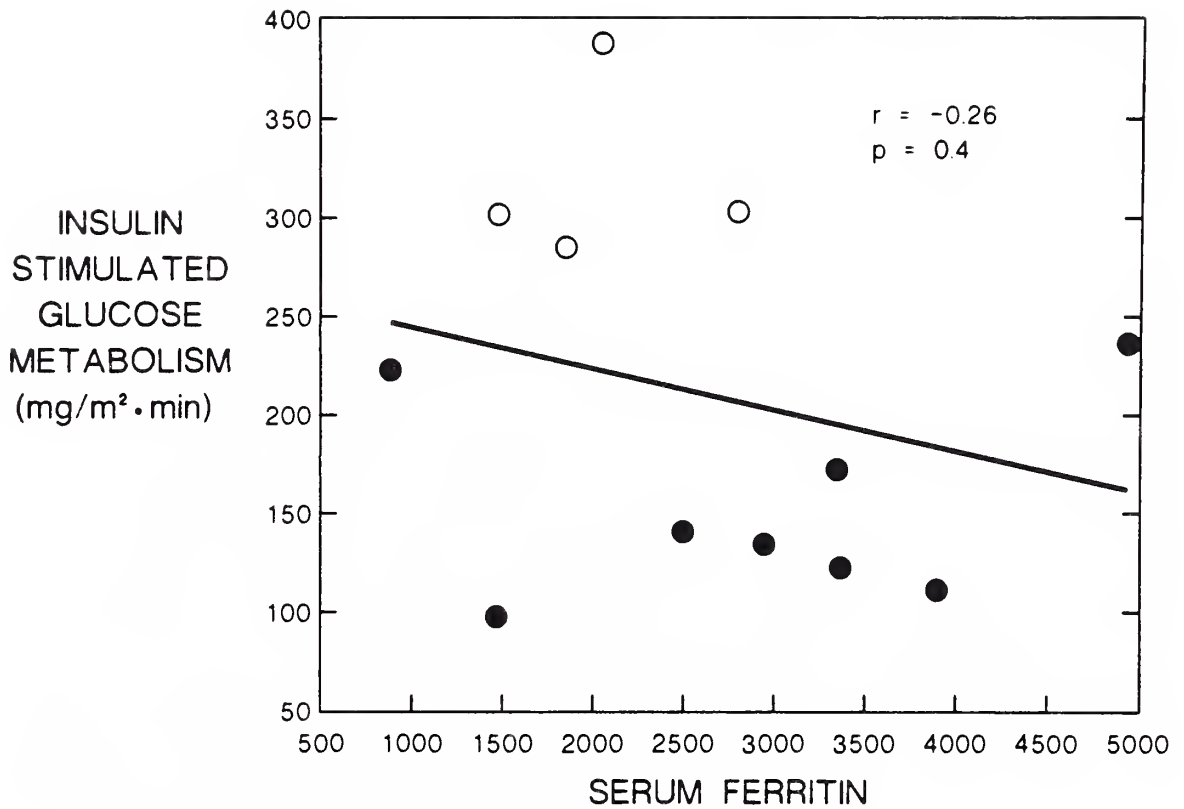
Figure 3: Relationship between insulin-stimulated glucose metabolism, as determined by the euglycemic insulin clamp procedure, and serum ferritin levels in patients with thalassemia major.
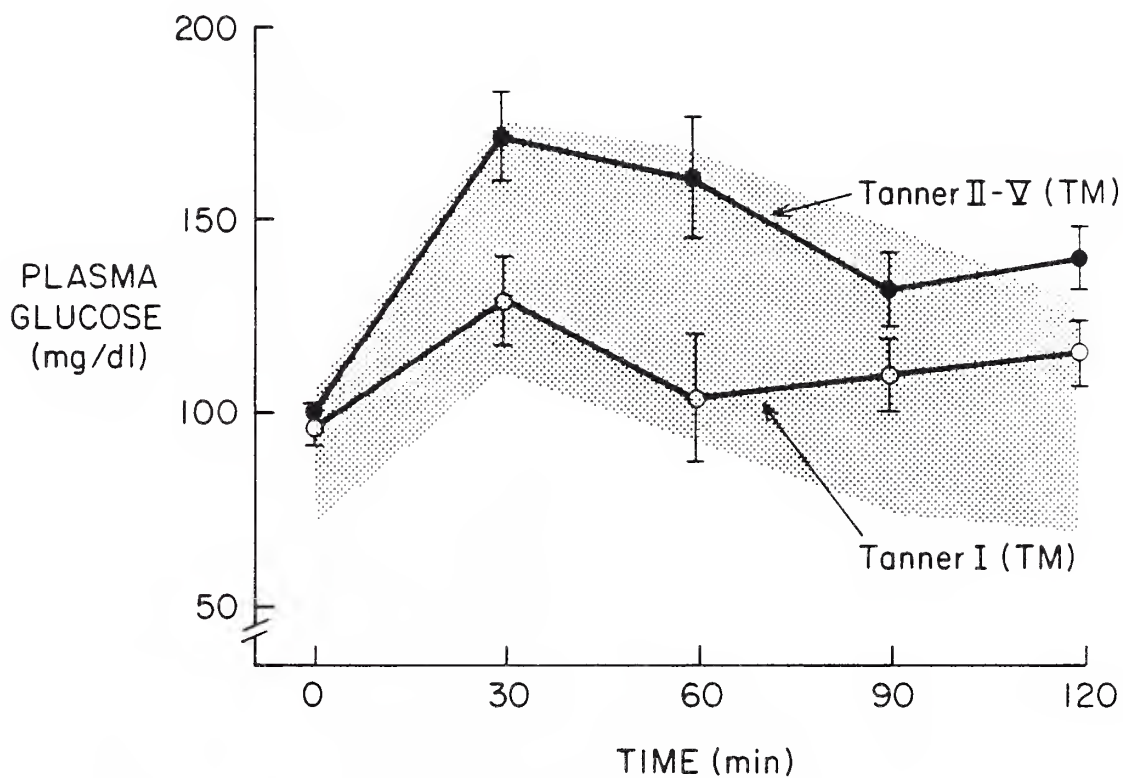
Figure 4: Plasma glucose levels (means ± standard error) during oral glucose tolerance testing in pubertal, Tanner II-V thalassemics (closed circles) and prepubertal, Tanner I thalassemics (open circles). Normal control range of values is indicated by the shaded area.
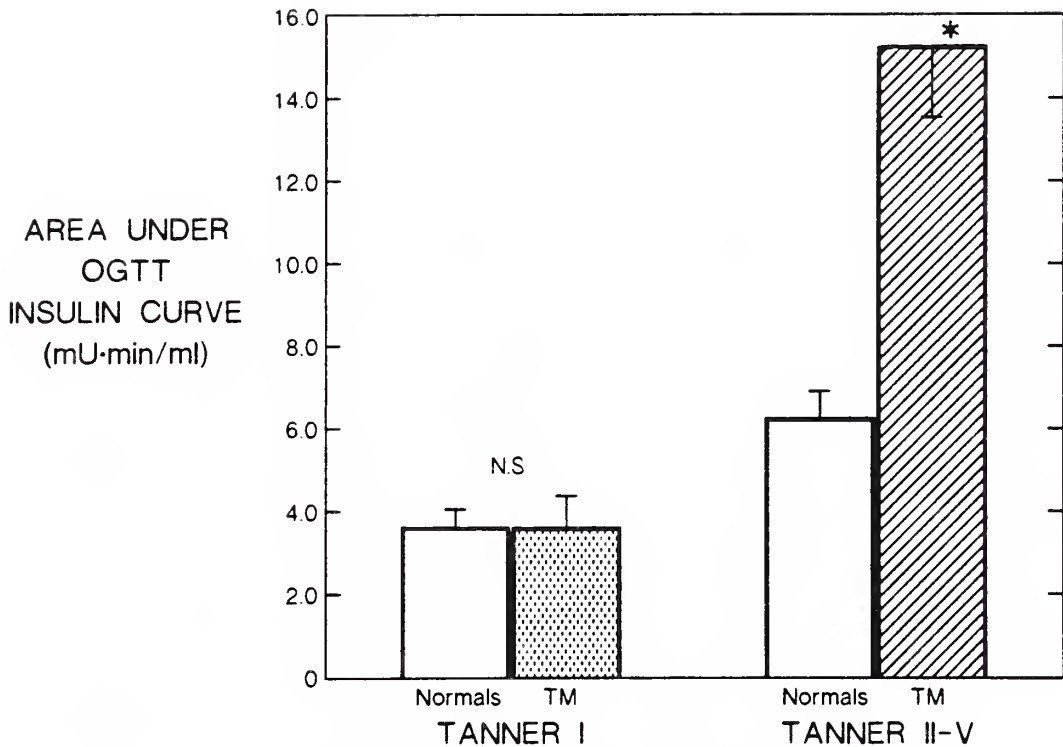
Figure 5: Area under the insulin response curve during oral glucose tolerance testing in Tanner I (stippled bars) and Tanner II-V (hatched bars) thalassemics (TM) and age-matched normal controls (open bars). Asterisk indicates significant difference between pubertal thalassemics and pubertal controls, p<0.001.
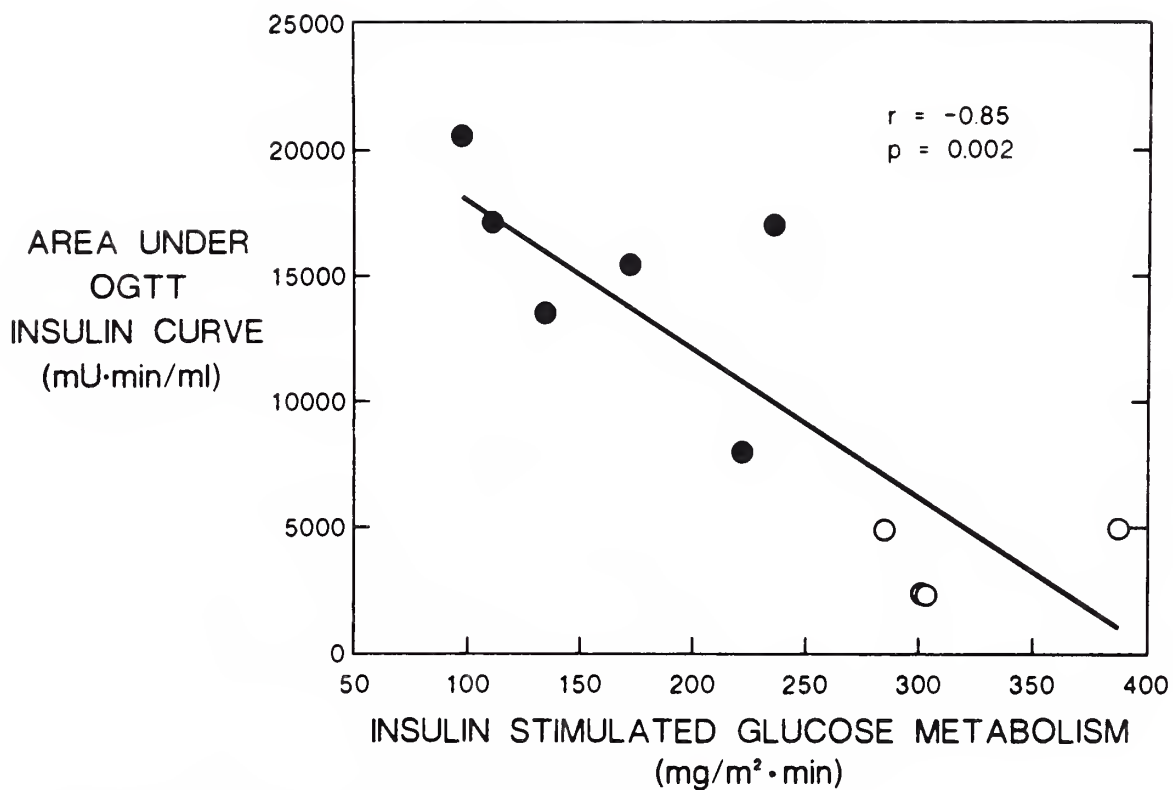
Figure 6: Correlation between the area under the insulin response curve and the rate of insulin-stimulated glucose metabolism as determined by the euglycemic insulin clamp procedure for all thalassemic subjects.
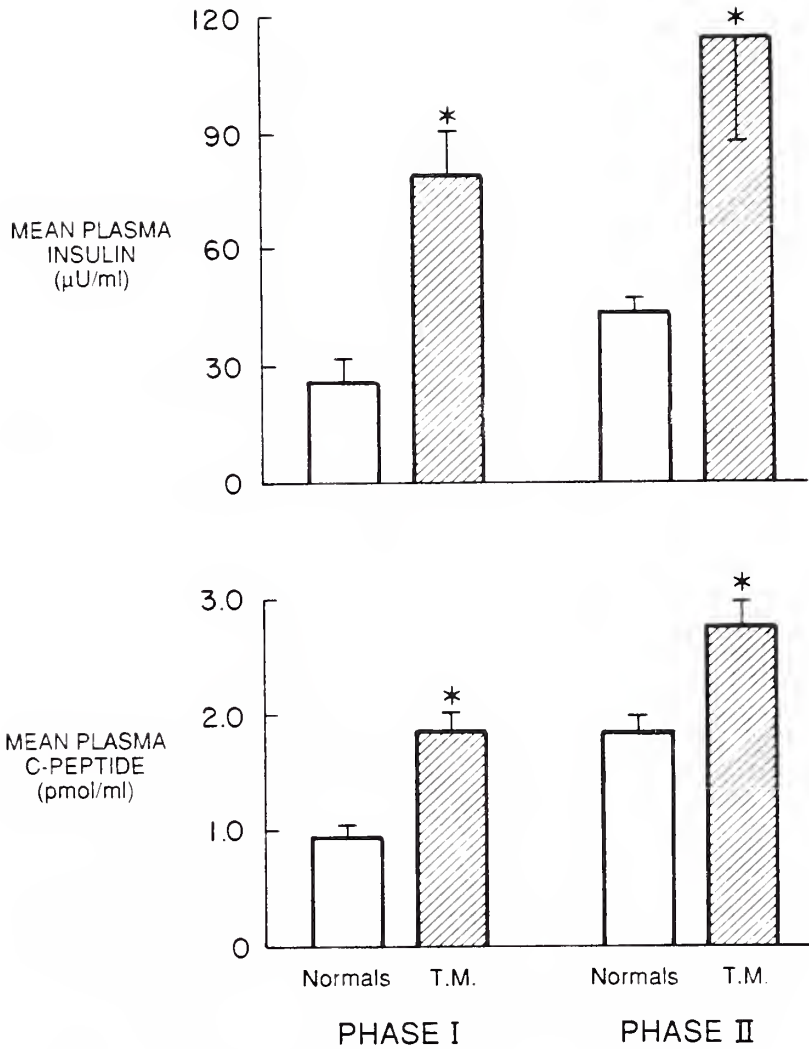
Figure 7: First and second phase plasma insulin (upper panel) and C-peptide (lower panel) responses during the hyperglycemic clamp study in pubertal thalassemics (TM, hatched bars) and pubertal control subjects (open bars). Asterisk indicates significant difference vs. corresponding value in normal controls, p<0.01.
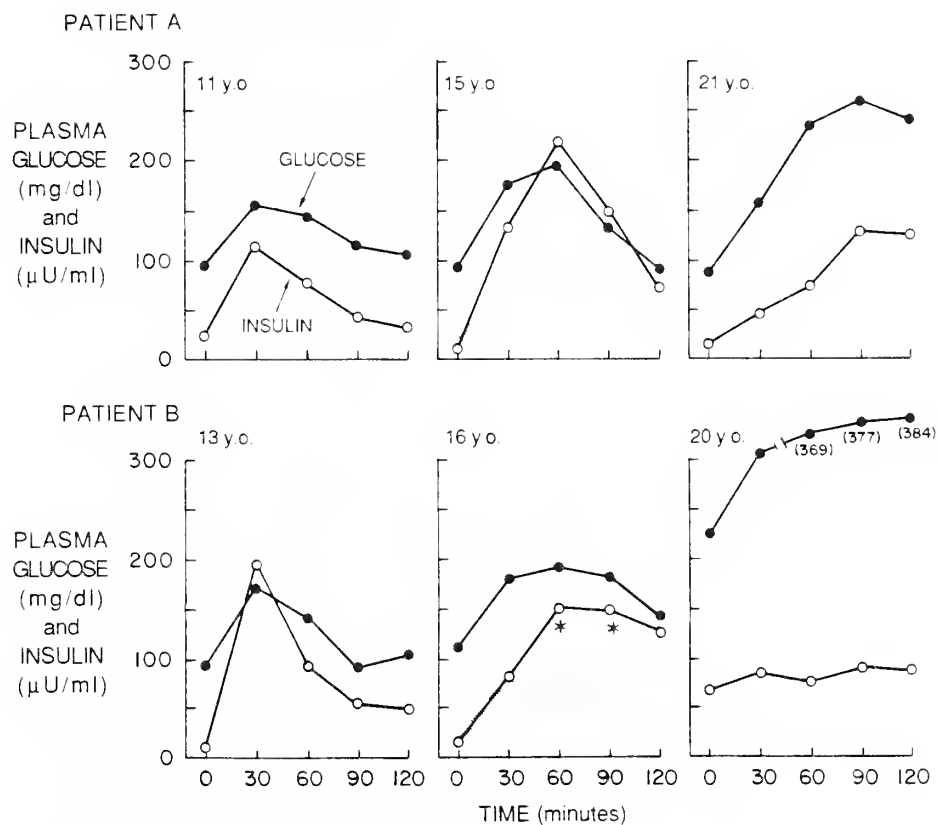
Figure 8: Plasma glucose (closed circles) and insulin (open circles) levels during sequential oral glucose tolerance testing in two female thalassemic patients who have developed diabetes. Insulin levels marked with an asterisk (Patient B) were reported as ≥150 μU/ml (1080 pmol/l); actual values may have been greater. Shaded areas in middle panels indicate plasma insulin values (means ± standard error) in normal pubertal control subjects.

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