Defining the criteria for Reference Interval calculation of selected Clinical Biochemistry analytes for identification of predisposition to disease and their risk calculation

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BY

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Saritha Francis

Dedicated to my dear
Parents
Husband
&
Children

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Abbreviations

ADA	American Diabetic Association
Ala	Alanine
ALT	Alanine Aminotransferase
BSA	Bovine serum albumin
CI	Confidence interval
CLSI	Clinical and laboratory standards institute
C peptide	Connecting peptide
CPH	Carboxypeptidase H
CRP	C-reactive protein
CV	coefficient of variation
Cys	cysteine
DAG	diacylglycerol
DNA	Deoxyribo Nucleic Acid
DM2	type 2 diabetes mellitus
FH	Family History of type 2 Diabetes mellitus
FSIVGTT	Frequently Sampled Intravenous Glucose Tolerance Test
G/I ratio	Glucose/insulin ratio
GIR	Glucose Infusion Rate
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose Transporter 4
HGP	Hepatic Glucose Production
His	Histidine
HLA	Human Leukocyte Antigen
HOMA	Homeostasis model assessment
hsCRP	high-sensitivity C-reactive protein
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IGF-1	insulin -like growth factor-1
IGT	Impaired Glucose Tolerance
IL-6	Inter Leukine 6
Ile	Isoleucine
IQR	Inter quartile Range

IRR	insulin receptor-related receptor
IRS	insulin/IGF receptor substrate
$ISI_{Matsuda}$	Insulin sensitivity index-Matsuda
IST	Insulin suppression test
K-S	Kolomogorov - Smirnova
LDH	Lactate dehydrogenase
MCP-1	monocyte chemo tactic protein-1
MCP-1	monocyte chemotactic protein- 1
NAD+	Nicotinamide adenine dinucleotide, reduced
NADH	Nicotinamide adenine dinucleotide
OCD	Ortho-Clinical Diagnostics
OGTT	Oral Glucose Tolerance Test
OR	odds ratio
PA	phosphatidic acid
PCR	Polymerised chain reaction
PI3	phosphatidylinositol 3
PIP3	phosphorylation of phosphoinositides
PTB	phosphotyrosine binding
QUICKI	Quantitative insulin sensitivity check index
RER	Rough Endoplasmic Reticulum
S_1	sensitivity index
SD	Standard Deviation
TNF-α	Tumor Necrosis Factor- α

GENERAL INTRODUCTION

India represents a region with the fastest increase in the prevalence of type 2 diabetes mellitus and is predicted to increase upto 2030 and may be further (Wild et al.,2004; Whiting et al.,2011). The prevalence is increasing worldwide and also in India (Ramachandran et al.,1988,Mohan et al.,2007; Sadikot et al., 2004). Type 2 diabetes mellitus is a lifestyle disease with multiple genetic and environmental influences on the phenotype (Ramachandran et al, 2001). Insulin resistance, pancreatic beta cell insulin secretory dysfunction and metabolic changes are central to the pathogenesis and progress to type 2 diabetes (Khan, 2003; Gerich, 2003; Weyer et al 1999). There are a number of complex secondary clinical influences which also influence the pathogenesis of the disease and the diabetic state of the individual (Khan, 2003). The objective of this study is to develop partitioned baseline reference intervals that may be used clinically to evaluate the environmental build up on the genetic or familial background level of a parameter. Though initially a number of parameters were selected for the preliminary studies, the analysis became too complex and it was decided to settle with two parameters clinically related to type 2 diabetes mellitus, circulating plasma/serum insulin and C peptide. The former represents the insulinemia/hyperinsulinemia/hypoinsulinemia states and the latter represents the secretory function of the beta cells of pancreas. The environmental or familial or genetic build up on the baseline reference intervals of these two parameters may help in evaluating or predicting the progress to the disease state and disease state itself (Horowitz, 2012).

Objective of the initial project proposal is given below:

a) Calculating the reference intervals of selected Clinical Biochemistry analytes.
b) Defining the clinical, biochemical, environmental and hereditary criteria of the selected analytes for defining new reference intervals of individuals with and without predisposition to a disease state.
c) Investigation on the differences in the initial and new reference intervals for diagnosis, risk calculation or predisposition of disease states.

The following paragraphs described how these objectives were fulfilled. A number of parameters were initially analysed in the preliminary studies for their potential and clinical utility for the calculation of baseline reference interval. The baseline reference interval is the partitioned reference interval where the environmental and clinical influences were lowest. A number of parameters were

found to be difficult to partition and were also difficult to define a baseline status without subclinical influences.

After identifying insulin and C peptide as two potential targets for baseline reference interval calculation, it was important to reduce variances and secondary influences to attain a baseline status of these parameters, so that they can be partitioned. In the preliminary studies, fasting and post glucose load Oral Glucose Tolerance Test (OGTT) insulin and C peptide were analysed and evaluated for their clinical influences and for the reference interval calculations. It was observed that fasting insulin and fasting C peptide had the lowest coefficient of variation and were found to be correlating with a number of clinical parameters. It was later observed that fasting insulin and fasting C peptide could be partitioned and sub partitioned according to family history of type 2 diabetes mellitus and gender. The reference intervals calculated were also found to be clinically useful, especially for fasting insulin. The reference interval calculation for fasting C peptide encountered difficulties. Fasting C peptide measures insulin secretion. The β cell dysfunction decreases insulin secretion, while insulin resistance increases insulin secretion (Khan, 2003; Gerich, 2003; Weyer et al 1999). These influences confounded each other and moderately influenced reference interval calculations.

The risk calculation for hyperinsulinemia performed well, but the risk calculation for fasting C peptide encountered problems due to the above mentioned confounding factors.

In chapter 3-6, fasting and post glucose load OGTT, insulin and C peptide were analysed for their correlations with parameters closely related to type 2 diabetes mellitus. They were also analysed for the influences by family history of type 2 diabetes and gender differences. It was possible to effectively partition fasting insulin and also fasting C peptide.

In chapter 7-9, the influence of family history of type 2 diabetes mellitus upto grandparents and the gender of the proband on fasting insulin and fasting C peptide were analysed. Fasting insulin could be partitioned into 3 groups according to family history of type 2 diabetes upto grandparents as groups I, II and III. C peptide could be partitioned according to gender in group II and group III with family history of type 2 diabetes.

In chapter 10 attempts were made to calculate reference interval of the groups that could be significantly partitioned. When the sample number was high above 120,

non parametric percentile based method was used to calculate the central 95% reference interval. When parametric methods were used to calculate 95% reference interval, extreme care was taken in transforming the data into Gaussian distribution. For this purpose, Box-Cox transformation was preferred. Extreme care was also taken to analyse the data distribution by use of methods such as Shapiro-Wilk test, Anderson - Darling normality test and D'Agostino – Pearson tests.

In chapter 11 odds ratio was used to calculate the risk of hyperinsulinemia, and hyperinsulinemia was related to family history of type 2 diabetes (Haffner, 1988; Elbein, et al 1999). The risk calculation with fasting C peptide was partially successful in group I and II but not in group III. In group III, beta cell dysfunction interfered with and confounded the increase in insulin secretion resulting from insulin resistance. To conclude, calculation of partitioned baseline reference interval calculation for fasting insulin was successful. This is significant because it could be used for clinical analysis of environmental build up on the phenotype with age, gender and lifestyle that lead to type 2 diabetes mellitus. But the calculation of baseline reference interval for fasting C peptide before and after partitioning according to gender could be done. But the risk calculation was successful for C peptide in groups I and II but not for group III. The risk calculation for hyperinsulinemia in group I, II and III, and with gender appears to be clinically significant and correlates with the prevalence data.

Chapter 1

REVIEW OF LITERATURE

1.1. Introduction to Review of Literature

The prevalence of type 2 diabetes mellitus is increasing worldwide and in India. India represents a region with the fastest increase in the prevalence and is predicted to increase upto 2030 and may be further. Type 2 diabetes mellitus is a lifestyle disease with multiple genetic and environmental influences on the phenotype. Insulin resistance, pancreatic beta cell insulin secretory dysfunction and metabolic changes are critical in the pathogenesis and progress to type 2 diabetes. There are a number of complex secondary clinical influences which also influence the pathogenesis of the disease and the diabetic state of the individual. The objective of this study is to develop partitioned baseline reference intervals that may be used clinically to evaluate the environmental build up on the genetic or familial background level of a parameter. Though initially a number of parameters were selected for the preliminary studies, the analysis became too complex and it was decided to settle with two parameters clinically related to type 2 diabetes mellitus, circulating plasma/serum insulin and C peptide. The former represents the insulinemia/hyperinsulinemia/hypoinsulinemia states and the latter represents the secretory function of the beta cells of pancreas. The environmental or familial or genetic build up on the baseline reference intervals of these two parameters may help in evaluating or predicting the progress to the disease state and disease state itself. The topics related to these aspects with reference to type 2 diabetes mellitus are reviewed in this section.

1.2. Epidemiology of Type 2 Diabetes Mellitus

1.2.1. Prevalence Worldwide and in India

The International Diabetes Federation (IDF) estimated that there are 100 million people with diabetes worldwide. This figure is expected to reach around 240 million by 2010. In Asia, prevalence of diabetes is higher and it has been estimated that 20% of the current global diabetic population resides in South-East Asia. Indeed, the number of cases in India is likely to double in two decades that is from 40 million (in 2007) to 70 million by 2025 (Ramachandran & Snehalatha, 2009).

Prevalence of diabetes in India showed an increase, as reported from 1988 onwards and was predicted to increase further (Ramachandran et al., 1988, Mohan et al., 2007; Sadikot et al., 2004). In the year 2000, India had the highest number of individuals with diabetes, and was predicted to increase fastest upto 2030 (Wild et al., 2004; Whiting et al.,2011). Prevalence was higher in urban when compared to rural population (Ramachandran et al.,1988;Vijayakumar et al.,2009; Ramachandran et al.,1992; Ramachandran et al.,2001). High prevalence of diabetes was reported in Kerala state, from where this study originates (Vijayakumar et al., 2009). There was increase in the prevalence of diabetes with age upto 60 - 69 years in urban population. Thereafter, the prevalence decreased with increase in age above 69 years. This was seen when the prevalence was 8.6% to 19.5% in different states of India from 2001 to 2006 (25 Mohan et al., 2007). In another report from Australia, with a prevalence of 4.4% (Collins & Kalisch, 2011), the prevalence increased upto 55 – 59 years of age and thereafter decreased. With increase in prevalence of diabetes, there is a progressive decrease in the average age of onset of diabetes in India (Mohan et al.,2007). A report from South India on familial aggregation of type 2 diabetes showed that there was absence of excess maternal transmission (Viswanathan et al.1996).

1.2.2. Effect of Urbanisation

Urbanisation and industrialisation have resulted in changes on the social and economic front in developing countries such as India. These have resulted in lifestyle changes leading to lifestyle related diseases. Transition from a traditional to modern lifestyle, consumption of diets rich in fat and calories, combined with a high level of mental stress has compounded the problem further. There are several studies from various parts of India which reveal a rising trend in the prevalence of type 2 diabetes in the urban areas. A National Urban Survey in 2000 observed that the prevalence of diabetes in urban India in adults was 12.1 per cent per cent (Ramachandran et al, 2001). Recent data has illustrated the impact of socio-economic changes occurring in rural India. The transition has occurred in the last 30 years and the prevalence has risen from 2.4 per cent to much higher levels of nearly 20% in some parts of India (Vijayakumar et al., 2009; Ramachandran et al., 1992; Ramachandran et al., 1988).

1.2.3. Variation according to age

The development of type 2 diabetes mellitus is profoundly influenced by attained age. The effect of age on risk to type 2 diabetes mellitus has been demonstrated in many cross-sectional studies. One of the best data on the prevalence of diabetes is available from NHANES III, conducted between 1988 and 1994. In all races men and women had similar prevalence of diabetes. The overall prevalence of diagnosed diabetes was 5.1% in adult US population. (Resnick et al, 2000). Indians develop diabetes at a very young age, at least 10 to 15 years earlier than the western population. An early occurrence of diabetes gives ample time for development of the chronic complications of diabetes. The incidence of diabetes increases with age. In India, the life span has increased; hence more number of people with diabetes is being detected.

1.2.4. Variation according to obesity

Studies showed that the risk of developing diabetes was higher in obese than non-obese individuals (Colditz et al, 1995). In a study of the 14 years follow up of Nurses' Health study, it was shown the incidence rate increases from 13 to 104/1,00,000 persons comparing women whose BMI <22 kg/m^2 to those with BMI in the range 25 – 26.9 kg/m^2. Both a high weight before age 18 years and a large weight gain during adulthood contributed significantly to the risk of type 2 diabetes mellitus. However, BMI significantly impact on the risk of type 2 diabetes mellitus the better predictor was waist-hip ratio or waist circumference. The studies also showed that BMI is a strong predictor for males than the waist circumference. The cut-off limit for waist circumference for Indians is 90 cm for males and 80 cm for females.

1.2.5. Variation according to physical activity

There is an inverse relationship between physical activity and the risk of type 2 diabetes mellitus. The dose response between intensity of physical activity and the risk of type 2 diabetes mellitus was estimated in many studies. In three studies in men the risk of type 2 diabetes mellitus decreased with increasing amount of exercise, whereas in women the degree of protection against type 2 diabetes was the same in subjects who exercised only moderately. All studies showed that individuals with a modest level of exercise had a significantly lower risk of type 2 diabetes mellitus than those who were completely sedentary (Manson

et al, 1992). There is enough evidence to demonstrate that physical inactivity as an independent factor for the development of type 2 diabetes. The availability of motorized transport and a shift in occupations combined with the plethora of television programmes has reduced the physical activity in all groups of populations. The impact of stress both physical and mental along with lifestyle changes has a strong effect of increasing incidence of type 2 diabetes amongst persons is a strong genetic background.

The occurrence of type 2 diabetes mellitus has been increasing in the world population over the last 50 years. The best incidence data was obtained from the study in the population of Rochester, Mimmesota (Leibson et al, 1997). This study represents an increase in 90% in man and 77% in women during the time period of 1945-1989. Another study during the period 1980-1987, the incidence rates of diagnosed and undiagnosed type 2 diabetes mellitus increased in Mexican Americans and Non-Hispanic Whites by 95% and 63%, respectively.

1.2.6. Variation among Races and Countries

The geographic and ethnic differences in the occurrence of type 2 diabetes mellitus can be used to evaluate environmental as well as genetic determinants of diabetes.

There is a large variability in the occurrence of type 2 diabetes mellitus according to racial/ethnic group. Large variability in the occurrence of type 2 diabetes mellitus within the same racial/ethnic group (e.g. Chinese) according to geography may be accounted for by different frequencies of environmental factors, such as physical activity and obesity in each of these locations. On the other hand, the very high prevalence of type 2 diabetes mellitus in Native Americans and in Indians may be due to interaction between a high population frequency of genetic susceptibility to type 2 diabetes mellitus and very high prevalence of environmental factors such as obesity physical inactivity or other risk factors.

Gender differences were reported in the prevalence of type 2 diabetes with paternal and maternal familiality of type 2 diabetes in Mexican Americans and non Hispanic whites from the San Antonio Heart Study, and in participants who are primarily Caucasians in the Framingham Offspring Study (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000).

In the year 2000, India had the highest number of individuals with diabetes, and was predicted to increase fastest upto 2030 (Wild et al., 2004; Whiting et al.,2011).

1.2.7. Clustering of type 2 diabetes mellitus in families

Type 2 diabetes mellitus has a complex etiology involving both environmental exposures and genetic susceptibility. For the evaluation of the role of genetic susceptibility, one has to obtain specific information about the pattern of aggregation of diabetes in families with type 2 diabetes mellitus. A widely used measure of familial aggregation is the sibling recurrence-risk ratio (λ/s) which is defined as the ratio of the risk of disease manifestation in siblings of cases compared with the disease risk in the general population (Penrose, 1953). Ratio above unity suggests familial aggregation.

NHANES III and all other studies based on recurrence risk ratio showed that the cumulative risk of diabetes in sibling/offspring of index cases has a steady increase or continuous increase with parents without diabetic, one diabetic parent and two diabetic parents. These studies also shows that sibling/ offspring of index cases without a history of parental diabetes had a risk of diabetes similar to that in the general population, suggesting that the contribution of a general component was inconsequential in this subgroup.

The λ/s of type 2 diabetes mellitus was high if a parent and grandparent reported diabetes. The λ/s is same for non-diabetic family history and for the family history of diabetes, as affected to grandparents, aunt or uncle instead of parents. Therefore, the risk of diabetes may be transmitted primarily through affected rather than unaffected parents. Some studies showed that age is not much influenced to genetic susceptibility. A young age at diagnosis of diabetes was itself associated with a history of diabetes in one and both parents. Obesity in the index case was associated with a reduced recurrent-risk of type 2 diabetes in siblings. Epidemiologic observation conducted that obesity and family history of diabetes are independent risk factors for the development of type 2 diabetes mellitus.

The genetic factors have led to clustering of type 2 diabetes in families (Gottlieb, 1980; Li et al, 2000; Hilding et al, 2006), its heritability (Hilding et al 2006; Almgren et al, 2011) and the high degree of concordance of diabetes in

monozygotic twins (Poulsen et al, 1999; Kyvik et al, 1995). There are racial and ethnic differences in the prevalence, insulin sensitivity, insulin response and risk of diabetes. There are also differences in the prevalence of diabetes and impaired glucose tolerance according to maternal or paternal history of diabetes (Kodama et al, 2013; Mitchell et al, 1993; Valdez et al, 2007). There is correlation between prevalence of type 2 diabetes and genetic admixture in hybrid populations (Serjeantson et al, 1983; Brosseau et al, 1979).

Increase in the prevalence of type 2 diabetes, impaired glucose tolerance and hyperinsulinemia (Gottlieb, 1980; Li et al,2000; Mitchell et al; 1993; Valdez et al, 2007) in offspring of parents with type 2 diabetes has been reported. Gender differences in the prevalence of type 2 diabetes in the offspring, related to paternal and maternal familiality or family history of type 2 diabetes have also been reported (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000). But what was not reported and the objective of this study are the gender differences in familiality and in fasting insulin of offspring, as follows: Partitioning of a young adult proband or participant of this study according to their gender and family history of type 2 diabetes upto grandparents; Prevalence of type 2 diabetes and its gender differences in parents and grandparents of the partitioned participants; Gender difference in fasting insulin in these partitioned participants. These are required for future estimation of the risk of hyperinsulinemia and type 2 diabetes in the proband.

Gender differences in the prevalence of type 2 diabetes with paternal and maternal familiality of type 2 diabetes in Mexican Americans and non Hispanic whites from the San Antonio Heart Study and in participants who are primarily Caucasians in the Framingham Offspring Study had been reported earlier (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000). The four major findings in the report by Mitchell et al, 1993 were: Men with parental history of type 2 diabetes had higher prevalence of diabetes and impaired glucose tolerance than men without parental history; In men, the prevalence was equally high regardless of which parent or both parents; Women with only a maternal history of type 2 diabetes was associated with higher prevalence of type 2 diabetes and impaired glucose tolerance; In women, there was no difference in the prevalence of type 2 diabetes between women with a paternal history of diabetes and women with no parental

history of diabetes. The Framingham Offspring Study showed that offspring with maternal diabetes and an age of onset of less than 50 years had increased risk of diabetes (Meigs et al, 2000).

The prevalence of diabetes is higher among the offspring of diabetic mothers than among the offspring of non diabetic mothers or diabetic fathers. These observations have been made in clinical and experimental studies (Meigs et al, 2000; Kasperska-Czyzyk et al, 1996; Pettitt et al, 1988; Thomas et al, 1994; Han et al, 2007). Exposure in utero to a diabetic mother is associated with a higher risk of obesity, IGT and type 2 diabetes in offspring (Pettitt et al, 1988; Dabelea et al, 2000; Silverman et al, 1995). Insulin secretion has been shown to be abnormal in offspring of diabetic mothers (Aerts et al, 1988).

Familiality of type 2 diabetes may be due to genetic and non genetic factors (Doria et al, 2008; Han et al, 2007; Dabelea et al, 2000). Non genetic factors may be epigenetic factors or non epigenetic issues such as cultural, behavioural and environmental aspects specific to the family. Familiality may also result from intrauterine influences on fetal growth and development, which can lead to changes later in life (Dabelea et al, 2000; Silverman et al, 1995; Aerts et al, 1988). The intrauterine effects and mitochondrial inheritance may also contribute to maternal transmission of diabetes.

1.3. Insulin: Biosynthesis, processing and activity

Insulin is a protein produced by the β-cells of the islets of Langerhans in the pancreas. Insulin was the first protein hormone sequenced, first substance to be measured by radioimmunoassay, and the first compound produced by recombinant DNA technology for practical use. It is an anabolic hormone that stimulates the uptake of glucose into fat and muscle, promotes the conversion of glucose to glycogen or fat for storage, inhibits glucose production from liver, stimulates protein synthesis and inhibits protein breakdown.

1.3.1. Insulin Structure

Insulin was the first protein to have its entire primary sequence is determined by Frederic Sanger (Nobel Prize in chemistry in 1958) and coworkers (Sanger, 1959). Insulin is composed of two polypeptide chains that are linked to

one another by disulfide bond. The A- and B- chains of human, porcine and bovine insulin are composed of 21 and 30 amino acids respectively. The two peptide chains are covalently linked to one another by two cysteine disulfides, one between CysA7 and CysB7 and other between CysA20 and CysB19. An additional intrachain disulfide connects cysteine A6 and A11. Insulin structure is highly conserved in vertebrate evolution. Regions of invariability include the position of cys disulfide bridges, the amino- and carboxy-terminal of A-chain and certain hydrophobic residues at the carboxy terminus of B-chain. Mutation and chemical modifications at highly conserved positions diminish or abolish receptor binding potency and biologic activity.

The 3D X-ray diffraction patterns of insulin crystals were conducted simultaneously by Dorothy Hodgkin (Nobel Prize in chemistry in 1964) and coworkers, and the Peking Insulin Structure Group (Adams et al, 1969). The crystal structure of insulin has been refined to a resolution of 1.5 A^{o}. Within each unit cell, there are two zinc insulin crystals, six insulin molecules compose a hexamer around two zinc atoms; three HisB10 residues chelate each zinc atom. Two zinc insulin hexamers can be subdivided into three equivalent dimmers, with each dimer being composed of two insulin molecules of similar but not identical structure. The hydrophobic core of the insulin monomer is composed of invariant and highly conserved residues, including leucine residues A16, B6, B11, and B15, the A6-A11 cysteines, the TyrA19 ring, IleA2 and AlaB14, supporting the notion that core structure must be maintained for insulin action. These core residues with hydrophobic side chains contact additional, more peripheral nonpolar residues including ValA3, LeuA13, ValB12, ValB18 CysB19 and PheB24, which in turn contact the remaining nonpolar residues (PheB1, ValB3, LeuB17 and PheB25). The more peripheral nonpolar residues that are exposed partially on the monomer surface contribute to the surfaces of insulin involved in protein-protein interactions, including the dimer and hexamer interfaces and possibly the receptor binding surface as well. Residues from both A- and B- chains comprise the hydrophobic core of insulin and the various binding surfaces. The A-chain form two helical segments (A1-A8 and A13-A19), which are connected by a turn; the B-chain contains two regions of extended chain (B1-B8 & B21-B30) connected by a region of α-helix (B9-B19). The two chains are connected to one another

covalently by cysteine disulfides and the overall structure is stabilized by interchain hydrogen bonds and ionic interactions between the N-terminus of the A-chain and the C-terminus of the B-chain.

1.3.2. Biosynthesis of Insulin

The biosynthesis and processing of the insulin molecule along the secretory pathway of the β-cell is a highly regulated and dynamic process (Steiner et al, 1967). It is evident that there is a protein precursor for proinsulin, preproinsulin, with the first 24 amino acids forming a signal peptide. This signal peptide translocates the nascent protein to the lumen of rough endoplasmic reticulum. The hydrophobic residues present in the signal peptide help in the penetration of RER membrane. In the RER the signal peptidase cleaves the signal peptide and thus the intact proinsulin is formed. Thus formed proinsulin undergoes appropriate folding so that the disulfide linkage between A and B chain of insulin are correctly aligned. Properly folded proinsulin is then delivered to cis-golgi apparatus with the help of ATP-dependent transport vesicles in RER and β-cell's microtubules network, Ca^{2+}, guanosine triphosphate and certain cytosolic protein factors. Trafficking of proinsulin from cis stacks-via the medial–to the trans golgi apparatus is mediated with COP family coatomer protein vesicles. In the trans golgi network, the proinsulin is separated from other secretory and integral membrane proteins (Walter & Johnson, 1994).

Thus formed proinsulin is packaged in secretory granules for secretion in response to stimulus. These secretory granules in β-cells are coated with clathrin molecules. The maturation of granules involve three parallel events; loss of clathrin, progressive acidification and proinsulin conversion.

1.3.2.1. Conversion of Proinsulin to Insulin

Two Ca^{2+} regulated endopeptidases are needed for the conversion of proinsulin to insulin – namely type I and type II. Type I cleave at the Arg31, Arg32 site of proinsulin and type II at Lys64 & Arg65 site. Type I endopeptidase activity was equivalent to prohormone convertase 3, PC3 (also known as PC1) and type II is equivalent to PC2. A third proteolytic enzyme is also involved in proinsulin processing is an exopeptidase, carboxypeptidase H (CPH). It is a Ni^{2+}-requiring exopeptidase with acidic pH optimum. Human proinsulin is sequentially cleaved

by PC3 at Arg31, Arg32 to generate des-31, 32 proinsulin, which is trimmed by CPH and then processed by PC2 at Lys64, Arg65 to obtain spilt 65, 66 proinsulin and finally trimmed by CPH to produce insulin, C-peptide and free basic amino acids (Orci et al, 1986).

1.3.3. Insulin Secretion

For insulin secretion two pathways are present – constitutive and regulated pathways. The constitutive pathway as the name implies, is not subjected to control by secretagogues. It occurs by the rapid transport of proteins from trans golgi to plasma membrane in small vesicles. These small vesicles fuse with the plasma membrane and the protein secreted to the extracellular environment. The regulated pathway involves the selective packaging of secretory proteins into secretory granules followed by secretion (exocytosis) in response to a stimulus.

Before the release of insulin via exocytosis, an insulin secretory granule must be transported from an intracellular storage pool to the β-cell surface. The β-cell is polarized and secretory granules appeared to be delivered to a certain location on the β-cell plasma membrane. The transports of insulin secretory granules are mediated via an interaction with the β-cell cytoskeleton framework of microtubules (microtubule associated proteins and motors e.g. (kinesin & dynein) and microfilaments. Specific signal is needed for the secretion; otherwise it is in the resting state. Insulin exocytosis is triggered by certain intracellular secondary signals, including an increase in cytosolic [Ca^{2+}]; and also by several secretory granules, cytosolic and plasma membrane proteins. Insulin exocytosis involves docking of insulin secretory granule with the plasma membrane and subsequent fusion by SNARE- hypothesis.

Human insulin (molecular mass 6000D) consist of 51 amino acids in two chains A & B, joined by two disulfide bridges, with a 3rd intra disulfide bridge within A chain. Preproinsulin (about 100 amino acids) is not detectable in circulation under normal conditions because it is enzymatically cleaved and converted to proinsulin. Proinsulin, which has relatively low biological activity, enters the circulation in small amount. In healthy individuals, at the time of fasting, approximately 5-10% of proinsulin and its intermediates are found in circulation. Proinsulin is cleared from the circulation less rapidly than insulin (because the

insulin receptor has lower affinity to proinsulin than insulin). This explains why the relative concentration of proinsulin in the periphery is higher than the portal vein.

1.3.4. C Peptide

Connecting peptide (C peptide), a cleavage product of proinsulin, is secreted by pancreatic β-cells in equimolar amounts along with insulin. This phenomenon has been exploited to assess prehepatic insulin secretion in humans. Unlike insulin, C-peptide is not significantly cleared by the liver and the kinetics of C peptide are linear at physiological and supraphysiologic plasma C peptide concentrations. Therefore, it has been suggested that peripheral C peptide levels more closely reflect pancreatic insulin secretion than do peripheral insulin levels.

Although a considerable amount of insulin is extracted by the liver, C peptide is subjected to negligible first pass metabolism by the liver, thereby serving as a surrogate marker for endogenous insulin secretion. C peptide has been considered an inert by-product of insulin synthesis and has also been of great value in the understanding of the pathophysiology of type 1 and type 2 diabetes mellitus. However, C peptide has recently been re-evaluated as a bioactive peptide in its own right.

It was believed for many years that C peptide was important only to ensure correct folding insulin and otherwise it was inactive. But now, studies showed that C peptide exerts direct effect on renal function, to augment glucose utilization and to improve autonomic nervous function in insulin dependent diabetes mellitus, as well as insulin secretion. All these effects could be mediated by direct impact of C peptide on Na^+K^+-ATPase activity in various tissues (Wahren et al, 2000). Although insulin and C peptide are secreted into the portal circulation in equimolar amounts, fasting concentration of C peptide are 5-10 fold higher than those of insulin due to longer half-life of C peptide (about 35 minutes). C peptide is removed from circulation by the kidneys and degraded with a fraction exerted unchanged in urine.

1.3.5. Regulation of Insulin Biosynthesis in the β-Cell

Nutrients, neurotransmitters and hormones control the rate of proinsulin biosynthesis. (Campbell et al, 1982) Glucose is the most physiologically relevant

among these. The metabolism of glucose generates intracellular signals to stimulate insulin biosynthesis. The threshold concentration of glucose required to stimulate insulin secretion is 4-6 mM (that stimulate insulin biosynthesis between 2-4 mM). Maximum proinsulin biosynthesis is reached at a glucose concentration of 10-12 mM. As well as glucose other sugars and nutrients, such as leucine, succinate, pyruvate, inosine, guanosine, adenosine and ribose are capable of stimulating insulin biosynthesis, but are less effective than glucose. When pancreatic islet β-Cells are exposed to a glucose stimulus, 20 minutes is required for significant increase in proinsulin biosynthesis. By 60 minutes, the rate is stimulated to 10-20 folds. The down regulation of proinsulin biosynthesis is relatively slow and takes one hour to return to basal rate, even after the removal of stimulus.

1.3.6. Crinophagy

If insulin is not released, it is degraded within the β-Cell by the fusion with lysosomes, which is known as Crinophagy (Smith & Farquhar, 1966). It was first shown to occur in pituitary mammotrophic cells and pancreatic β-Cells. Insulin is degraded slowly even after its introduction into lysosomes, due to the stability of insulin crystals, which display an acidic milieu similar to that of secretory granules.

1.4. Insulin Action

The classical effect of insulin on glucose homeostasis is its ability to stimulate glucose transport in fat and muscle cells. This occurs via a translocation of GLUT4 glucose transporters from intracellular site to the plasma membrane. Insulin can regulate the glycogen/ lipid/protein synthesis, cell growth and differentiations and also some gene expression. The other functions of insulin are, it can regulate hormone production, secretory function and signal sensing in neural or endocrine cells, regulation of estrogen/androgen balance in ovarian granulose cells, promote vasodilatation in endothelial cells.

1.4.1. Insulin Receptor

The insulin receptor is a tetrameric protein consisting of two α-subunits and two β-subunits. This belongs to a subfamily of receptor tyrosine kinases that also includes

the insulin -like growth factor-1 (IGF-1) receptor and an orphan receptor called the insulin receptor-related receptor (IRR) (Zhang & Roth,1992). Two of the α-β dimmers undergo disulfide linkage to form the tetramer. The insulin receptor is widely distributed throughout the body.

Insulin receptor behaves as an allosteric enzyme in which the α-subunit inhibits the tyrosine kinase activity intrinsic to the β-subunit. Insulin binding to the α-subunit, or removal of α-subunit by proteolysis or genetic deletion, leads to a derepression, i.e. activation of the kinase activity in the β-subunit. This initial activation follows the transphosphorylation of the β-subunit, i.e. one subunit phosphorylates the other, leading to a conformational change and a further increase in the activity of the kinase domain. The α- β-heterodimers of insulin, IGF-1 and the IRR receptors can form functional hybrids in which occupancy of one receptor's binding domain leads to activation of the other receptor in the heterodimer by this transphosphorylation process. A dominant negative form of one of these receptor subtypes can inhibit the activity of other receptors by forming heterodimers. This may explain why individuals with mutations in the insulin receptor exhibit both insulin resistance and growth retardation.

The insulin/IGF-1 signaling system is evolutionarily very ancient. In the lower organisms, this system uses many of the same downstream signals used in mammalian cells, i.e. phosphatidylinositol 3-kinase (PI3- kinase)/Akt/forkhead transcription factors. In drosophila, the insulin secretory cells are neurons. In *C. elegans*, a major effect of insulin/IGF system is aging, i.e. mutation in insulin receptor causes reduction in insulin action and thereby the life span becomes longer than normal.

There are nine intracellular substrates of insulin receptor tyrosine kinases are identified. Four of these are belong to the family of insulin/IGF receptor substrate (IRS) proteins. These IRS proteins have pieckstrin homology (PH) and phosphotyrosine binding (PTB) domains near the N- terminus accounts for high affinity for the insulin receptor. The molecular mass of IRS proteins ranges from 60-180kDa. Four IRS proteins are IRS1, 2, 3 and 4. IRS1 and 2 is widely distributed. In humans IRS3 gene appears to be non-functional. The other substrates include Gab-1, $p62^{dok}$, cbl and various isoforms of Shc.

Following insulin stimulation, the receptor directly phosphorylates most of these substrates on multiple tyrosine residues. The phosphorylated tyrosine in each substrate occur in specific sequence motifs and once phosphorylated serve as 'docking sites' for intracellular molecules that contain SH2 (Src-homology 2) domains. The SH2 proteins that bind to phosphorylated IRS proteins fall into two major categories. The best studied category is the adapter molecule, such as PI3-kinase or Grb2 molecule, which associates with SOS to activate the Ras-mitogen activated protein (MAP) kinase pathway. The other major category of proteins that binds to IRS proteins is enzymes, such as phosphotyrosine phosphatase, SHP2 and cytoplasmic tyrosine kinases, such as Fyn (King & Johnson, 1985).

IRS proteins also undergo serine phosphorylation in response to insulin and other stimuli. In general serine phosphorylation appears to act as a negative regulator of insulin signaling by decreasing tyrosine phosphorylation of IRS proteins.

1.4.2. Phosphatidylinositol 3-Kinase Pathway

The exact mechanism by which PI3-kinase transmit the insulin signal appears to be multiple. PI3-kinase itself catalyses the phosphorylation of phosphoinositides on three positions to PI-(3)P, PI-(3,4)P2 and PI-(3,4,5)P3 (also known as PIP3). These lipids bind to the PH domains of signaling molecules and alter their activity or subcellular localization. PI3-kinase also activates the mTOR/FRAP pathway and activates phospholipase D, leads to the hydrolysis of phosphatidylcholine and increases in phosphatidic acid (PA) and diacylglycerol (DAG). Insulin also activates glycerol-3-phosphate acyltransferase, which increases de novo synthesis of PA and DAG (Cheatham et al., 1994).

The AGC superfamily of serine/threonine protein kinase is a major class of signaling molecule that are regulated by PI3-phosphatase, phosphorylates and activates the serine/ threonine protein kinase Akt [also known as PKB]. Akt/PKB plays an important role in transmission of insulin's metabolic pathway by phosphorylating glycogen synthase kinase and either directly or indirectly the forkhead (FOXO) transcription factors and the cyclic AMP regulatory element bind protein CREB.

1.4.3. Ras-mitogen activated protein kinase cascade

The second major pathway activated by insulin is the ras-mitogen activated protein kinase cascade (Myers et al, 1994). The tyrosine phosphorylation of IRS protein causes the binding of adapter protein Grb2, which recruits the guanyl nucleotide exchange protein SOS to plasma membrane. This causes the activation of Ras. Activated Ras operates like a molecular switch, which converts the upstream tyrosine phosphorylation into a second serine kinase cascade, via the stepwise activation of Raf, MAP-kinase-kinase MEK and the MAP kinase themselves, ERK 1 & 2. ERK1&2 can phosphorylates substrates in the cytoplasm or can translocate into nucleus and catalyze the phosphorylation of transcription factors such as $p62^{TCF}$, initiating a transcriptional program that leads the cell to commit to a proliferation or differentiative cycle.

PI3-kinase also stimulates the protein kinase mTOR (mammalian target of rapamycin) which is involved in protein synthesis/degradation and interaction with nutrient sensing. mTOR regulates the mRNA translation via phosphorylation and activation of p70 ribosomal S6 kinase and phosphorylates eIF-4E inhibitor, thus activating ribosome biosynthesis and increase in translation.

1.4.4. Turning off the Insulin Signal

To avoid the danger of hypoglycemia, there is a rapid on and off of insulin action on glucose homeostasis is demanded. Several mechanisms play a role in turning off of insulin signal (Kuhne et al, 1994). One such mechanism is the dissociation of insulin from the receptor and degraded. Following dissociation of ligand, phosphorylation of the insulin receptor and its substrates is rapidly reversed by the action of protein tyrosine phosphatase. Another mechanism involved in insulin turning off is the internalization of the insulin receptor itself and degraded. Serine phosphorylation of insulin receptor and its substrate also inhibit insulin action. The phosphorylated receptor may interact with proteins in the cell that block insulin action as in case of insulin resistance associated with inflammation and obesity.

1.5. Insulin resistance

Insulin is a peptide hormone secreted by the β cells of the pancreatic islets of Langerhans. It maintains normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and

promoting cell division and growth through its mitogenic effects. Insulin resistance is where a normal or elevated insulin level produces an attenuated biological response; classically this refers to impaired sensitivity to insulin mediated glucose disposal. Compensatory hyperinsulinaemia occurs when pancreatic β cell secretion increases to maintain normal blood glucose levels in the setting of peripheral insulin resistance in muscle and adipose tissue (Wilcox, 2005).

Insulin resistance syndrome refers to the cluster of abnormalities and related physical outcomes that occur more commonly in insulin resistant individuals. Metabolic syndrome represents the clinical diagnostic entity identifying those individuals at high risk with respect to the (cardiovascular) morbidity associated with insulin resistance (Reaven, 2004).

Insulin resistance can be divided into two – decreased insulin sensitivity (normal response but increased hormonal level) and decreased responsiveness (normal level of hormone but less response). Insulin resistance can be seen in type 2 diabetes, obesity, hypertension, polycystic ovarian disease and a variety of genetic syndromes, and in physiologic conditions such as puberty and pregnancy (Kahn, 1978). Insulin resistance is also present in many states of stress, in association with infection and secondary to treatment with a variety of drugs, particularly glucocorticoids. Insulin resistance plays an important role in type 2 diabetes and metabolic syndrome. In type 2 diabetes studies showed that there is a decrease in insulin receptor concentration, in receptor kinase activity, in the concentration and phosphorylation of IRS-1 and 2, in PI3-kinase activity, in glucose-transporter translocation and defects in activity of intracellular enzyme; but there is no reduction in the insulin action on MAP-kinase pathway (Reusch, 2002). Most longitudinal studies showed that Insulin resistance can be seen early in life before any evidence of glucose tolerance, whereas β-cell failure develops later, in association with IGT.

Studies showed that insulin resistance in muscle leads to increased accumulation of fat and secondary Insulin resistance, hyper triglyceridemia and increased levels of free fatty acids. Insulin resistance in liver leads to increased hepatic glucose output. Insulin resistance in brain leads to increased appetite, more obesity and further defect in hepatic glucose output. Finally Insulin resistance in β-

cell leads to defect in glucose sensing and thereby leads to relative insulin deficiency. Thus Insulin resistance in multiple tissues could produce all of the defects associated with type 2 diabetes and treatment that improves insulin sensitivity would be expected to improve all defects.

1.5.1. Pathogenesis of Insulin Resistance

In the pathogenesis of insulin resistance there is an interrelationship between genetic inheritance, obesity and environmental factors. Insulin resistance can occur at multiple levels and can be either acquired or genetic.

1.5.1.1. Acquired Form of Insulin Resistance

This may occur as a result of multiple mechanisms. Mild hyperinsulinemia occurs in response to tissue insulin resistance, which causes an increase in internalization and degradation of insulin receptor as in the case of obesity and type 2 diabetes (Gavin et al, 1974). Recent studies shown that hyperinsulinemia can lead to down regulation of insulin receptor substrates causing a greater decrease in insulin signaling. Hyperinsulinemia and other insulin resistance states shows an increase in serine phosphorylation of receptors and its substrates thus decrease in kinase activity receptor and tyrosine phosphorylation of receptor substrates.

1.5.1.2. Genetic Factors affecting Insulin Resistance

Insulin resistance due to genetic defect is the most severe form, including two congenital diseases termed leprechaunism and Rabson-Menhenhall syndrome and type A syndrome of insulin resistance (Taylor & Arioglu, 1998). A strong genetic basis for insulin resistance is suggested by the high prevalence of in certain populations, particularly the Nauru islanders of the pacific, the Pima Indians in Arizona and the urban Wanigela people in Papua New Guinea. Although the current epidemic levels of insulin resistance and type 2 diabetes in these populations have followed the introduction of “westernize” lifestyle characterized by high caloric intake and physical inactivity, their prevalence is much higher in these population than in populations of other ethnic groups with the same lifestyle. Furthermore, there is a nearly 100% concordance in diagnosis of type 2 diabetes between monozygotic twins but only a 20% concordance between dizygotic twins.

Extreme insulin resistance is linked to several rare mutations in genes associated with insulin action. Genetic conditions are associated with typical clinical manifestations such as hyperinsulinemia, dyslipidemia, hypertension, and IGT or insulin resistance diabetes. In women extreme insulin resistance is also associated with hyperandrogemism, hirsutism, menstrual abnormalities and polycystic ovarian diseases.

1.5.1.3. Free Fatty Acids and Intracellular Triglycerides in Insulin Resistance

Increased circulation of free fatty acids, play a central role in pathogenesis of insulin resistance. Free fatty acids inhibit insulin stimulated glucose uptake by glucose transport, inhibit glycogen synthesis, and also inhibit insulin stimulated glucose oxidation. Central obesity is associated with insulin resistance. Some central adipocytes are more resistant to insulin-inhibition of lipolysis, and thus an increase in the delivery of free fatty acids to liver. This causes the accumulation of triglycerides, which in turn increases hepatic glucose output, reduced hepatic extraction of insulin and thus hepatic insulin resistance. The link between increased free fatty acids and insulin resistance in type2 diabetes could be the accumulation of triglycerides in muscles.

1.5.1.4. Effect of Obesity on Insulin Resistance

Excess body fat or obesity is an important factor in the pathogenesis of insulin resistance and substantially increases the risk of type 2 diabetes (Vague, 1996). Now-a-days the prevalence in obesity is rising, the levels of physical activity are decreasing and there is a shift in diet occurred, including increased consumption of sugars and energy-dense food. This trend is seen all over the world. Global availability of cheap vegetable oils and fats has resulted in great increase of fat consumption among people in low income nations, including many Asian countries, Latin Americans, North Africa, Middle East and Urban areas of sub Saharan Africa. Dramatic increase in the consumption of low cost fat and simple carbohydrate calories resulted in steep increase in the incidence of overweight and obesity.

Adipose tissue is the body's principal site for energy storage and also influences whole body insulin action through the release of free fatty acids and

adipose derived proteins. Adiponectin (also known as Acrp30, AdipoQ & GBP28) is a 30kDa adipose specific secretory protein that appears to enhance insulin sensitivity. Decreased circulating level of this hormone accompanies obesity and insulin resistance in both humans and animal models. Adiponectin level is negatively correlated with hyperinsulinemia. Genetic polymorphism of adiponectin gene is also associated with increased incidence of insulin resistance and diabetes. Leptin is a 16kDa protein secreted from adipose tissues. This hormone is a member of cytokine family which acts on receptors in central nervous system and other sites to inhibit food intake and promote energy expenditure. Leptin also interfere with insulin signaling system in vitro. Leptin deficiency contributes to hyperinsulinemia and insulin resistance. Resistin is a 10kDa peptide hormone secreted by adipocytes. Initial studies shows that resistin levels were increased in both genetic and acquired obesity in mice. Administration of antibody to resistin appeared to improve blood glucose levels and insulin action in mice with diet-induced obesity. Thus we can conclude that there is a positive correlation between insulin resistance and resistin molecules.

Inflammation is also a causative of insulin resistance. Adipose tissues produce many pro inflammatory molecules, including TNF-α, IL-6, transforming growth factors-β, C-reactive protein and monocyte chemotactic protein-1 (MCP-1). These molecules are believed to induce systemic insulin resistance and to contribute to the pathogenesis of many metabolic complications of obesity, including type 2 diabetes and atherosclerosis. Intramuscular and intra hepatocellular triglycerides are also associated with insulin resistance in type 2 diabetes in humans.

1.5.1.5. Environmental Factors contributing to Insulin Resistance

Many medical conditions are reported to cause insulin resistance in some individuals. One of the first medical causes of insulin resistance to be identified was the presence of antibodies to insulin receptor (type B insulin resistance). Hepatic cirrhosis is also associated with glucose intolerance and insulin resistance. Iron overload appears to impair both hepatic and peripheral insulin action early in the course of hereditary hemochromatosis. Iron overload in thalassemia shows insulin resistance. Insulin resistance was also seen in several types of cancer,

particularly malignancies of gastrointestinal tract and pancreas. Hormonal excess also contribute to insulin resistance. Hyperinsulinemia is a classic indicator of insulin resistance. Increased sympathetic nervous system activity contributes to decreased insulin signaling. Excess of growth hormone blocks insulin actions. Dramatic lifestyle changes with unprecedented increase in nutrient intake now cause nutrient-induced insulin resistance.

1.5.1.6. Oxidative Stress and Insulin Resistance

Oxidative stress reduces insulin response and impairs insulin signaling. The antioxidant lipoic acid prevents the induction of insulin resistance in the presence of oxidative stress. It has been proposed that activation of common stress-activated signaling pathway such as nuclear factor-KB, p38 MAPK, and NH_2-terminal Jun kinases/stress-activated protein kinases by glucose and possibly free fatty acids leads to both insulin resistance and impaired insulin secretion. Increased nutrient availability increases the production of reactive oxygen species and thereby results in activation of PKC isoforms, increased formation of glucose-derived advanced glycation end-products, and increased glucose flux through the aldose reductase pathway.

1.5.1.7.Age and Insulin Resistance

The last "environmental" factor that contributes to insulin resistance is advancing age. (Ferrannini et al, 1997). Various characteristic features of aging that could predispose to insulin resistance include increased fat mass, particularly visceral adiposity, increased circulating level of inflammatory proteins and increased cellular accumulation of triglycerides. It has recently been proposed that an age-associated decline in mitochondrial functions contributes to insulin resistance in the elderly.

1.6. β-Cell Dysfunction in Type 2 Diabetes Mellitus

The initial event in the glucose intolerance is a genetic predisposition. Although specific polymorphism or mutated genes are not yet known, many that

affect the liver, skeletal muscle, adipose,β-cells or brain physiology will undoubtedly be uncovered. Lifestyle and environmental factors also determine the development of glucose intolerance (Hamman, 1992). β-cell dysfunction plays a crucial role in type 2 diabetes mellitus. Insulin secretion and tissue insulin resistance are of equal importance in the development of the disease. Type 2 diabetes is a progressive disease.

1.6.1. Normal β-Cell Function

The storage and metabolism of cellular fuels are regulated through secretion of insulin by pancreatic β-cells. The crucial function of β-cell is insulin secretion, proinsulin biosynthesis, processing of proinsulin to insulin and β-cell replication. The secreted insulin lowers glycemia by inhibiting hepatic and renal glucose production and increasing glucose uptake into target organs, primarily skeletal muscle. Glucose regulation of insulin secretion occurs directly (glucose induced insulin secretion) and also through modulation of insulin response to insulinotropic hormones, nutrients and neurotransmitters (glucose potentiation of nonglucose secretagogues). These dual aspects of glucose-regulated insulin secretion are a potent modulatory system that ensures that the tissue's need for insulin is exactly met in the fasting and postprandial states. The need for insulin is for the most part determined by the sensitivity of tissue to insulin - a curvilinear relationship exists with insulin secretion (Kahn et al, 1993).

An acute increase in glycemia elicits a large burst of insulin secretion that lasts for 5 to 7 minutes (first phase) followed by sustained insulin secretion that lasts for the duration of hyperglycemia (second phase). Meals also induce a biphasic pattern of insulin secretion, with the early phase ascribed to first 30 minutes and later phase to the remaining postprandial hyperinsulinemia (1 to 2 hours normally). Insulin secretion occurs as oscillatory pulsations with a periodicity of 11 to 14 minutes (Porksen et al, 1997). Also a large burst of insulin release (ultradian oscillations) occurs several times a day, particularly with meals (Polonsky et al, 1998), increasing the efficiency of nutrient clearance following meals.

Thus, the β-cell functions in a highly complex fashion that regulates the timing and overall insulin response to a meal to preserve normoglycemia. Insulin is

secreted into the portal vein and undergoes a substantial first pass clearance by liver. Thus, insulin levels in peripheral circulation only approximate insulin secretion. Now, many investigators analyze C-peptide values. C-peptide is secreted with insulin in an equimolar ratio but undergoes minimal hepatic degradation and thus can be used for calculating true rates of insulin secretion.

1.6.2. β-Cell Dysfunction in Type 2 Diabetes Mellitus

Mainly three types of dysfunction of β-cells can be seen at the time of type 2 diabetes mellitus. They are as follows

1.6.2.1. β-Cell unresponsiveness to glucose

β-cell defect in type 2 diabetes mellitus is the loss of the first phase of glucose-induced insulin secretion. Studies showed that this defect was fully established at the time when fasting glucose reached 115 mg/dL (Brunzell et al, 1976). This defect is an important cause of IGT state. Studies showed that in type 2 diabetes mellitus subject, intensive glycemic control restore the first phase insulin response, at the same time, glucose-induced insulin secretion was impaired. The understanding that evolved from these finding is that the defect in glucose-induced insulin secretion occurs when β-cells are exposed to a toxic environment of an abnormally high level of glycemia (glucose toxicity). Insulin responses to non-glucose secretogogues are less impaired than those to glucose. In type 2 diabetes mellitus the defective glucose regulation of insulin secretion is through two pathways – 1) glucose-induced insulin secretion (particularly in the first phase) and 2) glucose potentiation- emphasizing why fasting and postprandial hyperglycemia are defining of this disease. At the prediabetic/IGT state, there is a loss of early response results in an excessive meal induced rise in glycemia and this hyperglycemia causes the late insulin response to exceed that seen normally. Insulin levels, 2 hour after a meal or oral glucose challenge is not adequate to study the affect of early insulin secretion; a 30 minute insulin values are also needed. The loss of first phase insulin response to a meal is a characteristic feature of type 2 diabetes mellitus, occurs early in the course off the disease, predating fasting hyperglycemia and is a major cause of the postprandial hyperglycemia that characterizes IGT and overt diabetes. This concept underlies the recent use of drugs for type 2 diabetes mellitus (meglitinides and phenylalanine derivatives) that are taken at meals to induce a rapid, short-lived insulin response.

1.6.2.2. Abnormal pulsatile secretion of insulin

The pattern of orderly oscillation of insulin secretion with an 11- to 14-minute periodicity is lost in type 2 diabetes mellitus (Lang et al, 1981). This defect has a genetic etiology. Now, it is generally accepted that the abnormal insulin pulsatile impairs, normal regulatory control of insulin over hepatic glucose production. Glucagon-like peptide-1 (GLP-1), as a hypoglycemic agent for type 2 diabetes mellitus therapy, increases pulsatile insulin secretion. The ultradian oscillation is also disrupted in type 2 diabetes mellitus.

1.6.2.3. Increased proinsulin to insulin ratio

In type 2 diabetes mellitus, the blood levels of proinsulin are disproportionately increases relative to insulin (Temple et al, 1989). The finding showed that this disproportionate increase is a manifestation of hyperglycemia induced β-cell dysfunction. Some data showed that this increase in proinsulin/insulin ratio is also seen in the absence of glucose intolerance in population at high risk of type 2 diabetes mellitus. Treatment studies using somatostatin normalize the proinsulin/insulin ratio. The increased proportion of stored and circulating proinsulin in diabetic appears to be secondary to a hyper mobilization of granules, which leads to a rapid transit time and then to incomplete processing to fully mature insulin.

1.6.2.4. β-Cell Mass and Structure in Type 2 Diabetes Mellitus

The second abnormality caused to β-cell is the lowering of β-cell mass. Relatively little is known about this subject due to the unavailability of pancreatic biopsy samples from free-living humans. The study by Kloppel et al, 1985 is one of the largest studies observed a doubling of β- Cell mass in obese versus non-obese control subjects and a 50% reduction in β-Cell mass in obese and non-obese diabetic subjects. A recent finding in some diabetic animals showed that there is increased β-Cell apoptosis, although its relevance for human type 2 diabetes is unknown. This apoptosis is due to the development of islet amyloid in β-Cells of diabetic subjects.

1.6.2.5. Mechanism of β-Cell dysfunction

The main mechanisms of β-Cell dysfunction are glucose toxicity, β-Cell exhaustion and impaired proinsulin biosynthesis and lipotoxicity.

1.6.2.6. Glucose toxicity

Chronic hyperglycemia causes alterations in β- cell function, termed glucose toxicity. A universal finding is that secretion in response to glucose is impaired largely as opposed to secretion in response to non-glucose secretagogues such as arginine, glucagon or tolbutamide, which are relatively unaffected. This pattern of selective unresponsiveness to glucose closely resembles the pattern in human type2 diabetes. Glucose toxicity is the complete absence of β- Cell unresponsiveness to glucose. Many biochemical and molecular mechanisms have been proposed based on studies of islet tissue from diabetic animals.

1.6.2.7. β-Cell Exhaustion

β-cell exhaustion or overwork is a relative concept as β-cell dysfunction from a non-sustainable hyper stimulation of insulin secretion. In that case, hyperglycemia is the stimulus for the β-cell dysfunction but is not the operative mechanism. Many findings support a causative role for excessive insulin release in the defective glucose-potentiated secretion of insulin with chronic hyperglycemia. The concept is that a substrate, cofactor, cellular fuel, or other required substances is depleted, resulting in a lowered insulin response to meals.

1.6.2.8. Impaired Proinsulin Biosynthesis

The transcriptional control of proinsulin biosynthesis is impaired by chronic hyperglycemia and this causes lowering of insulin secretion in type 2 diabetes. Zangen et al reported impaired proinsulin transcription that paralleled lowered expression of PDX-1 in 90% of pancreatectomized diabetic rats (Zangen et al, 1996). A recent cross sectional study of diabetic rats with various levels of hyperglycemia suggests that inhibition of gene expression of proinsulin requires severe hyperglycemia. This effect plays a role in β-cell dysfunction of markedly hyperglycemic type 2 diabetes but not in the β-cell failure of new-onset diabetes, in which hyperglycemia is typically mild for most patients.

1.6.2.9. Lipotoxicity

Beside hyperglycemia other metabolic disruptions cause diabetic environment, including hypertriglyceridemia and elevated circulating and tissue levels of free fatty acids. Fatty acids lower the expression of IDX-1 (also called PDX-1) which is a key transcription factor for β-cell development. Free fatty acids impair glucose oxidation by lowering the activation of pyruvate dehydrogenase (Randle cycle). This way lipid can induce β-cell dysfunction (Zhou & Grill, 1995).

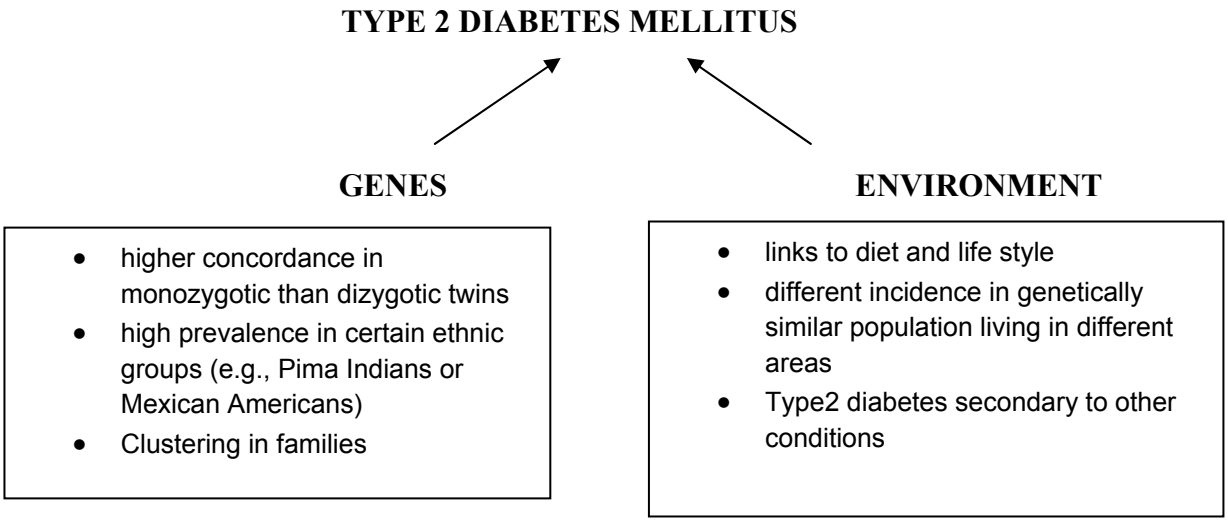
The prospective studies of the progression to type 2 diabetes have highlighted the crucial role played by β-cell dysfunction. The individuals whom diabetes developed, defect in both insulin secretion and insulin action were present when they were at normoglycemic, but it was the lowering of insulin response to intravenous glucose correlated with the progression from normoglycemia to diabetes. It is now clear that once diabetes develops, the β- cell dysfunction contributes to exert a major influence.

1.7. Genetics of Type 2 Diabetes Mellitus

The contribution of genetic factors to the development of insulin resistance, impaired insulin secretion, and type 2 diabetes has been known for many years (Harris, 1950). Supporting evidence includes the familial clustering of these traits, the higher concordance rate of type 2 diabetes mellitus in monozygotic versus dizygotic twins, and high prevalence of type 2 diabetes in certain ethnic groups (e.g. Pima Indians or Mexican Americans). On the other hand, environmental factors also appear to play a role, as indicated by the increasing incidence of type 2 diabetes during past decade, the well-known links to diet and life style, and the differences in risk of type 2 diabetes among genetically similar populations living in different areas. Thus, diabetes is a complex disorder, with genetic factors conferring an increased susceptibility upon which environmental factors must act in order for hyperglycemia to develop. It is estimated that between 25% and 70% of the occurrence of type 2 diabetes can attributed to genetic factors (Poulsen et al, 1999).

Genetic susceptibility and environmental factors may interact in several ways for the progression of type2 diabetes mellitus. Environmental factors such as

excess caloric intake or sedentary lifestyle may be responsible for the β-cell damage, while genetic factors may be involved in regulating the rate of progression to overt diabetes. Because of the important role played by environmental factors, cases of type2 diabetes that are due to entirely too nongenetic causes (phenocopies) may be relatively frequent, especially in older populations. Conversely, diabetes may not develop in a substantial proportion of individuals carrying a susceptible genetic background because they have not been exposed to a diabetogenic environment or because they are not old enough (nonpenetrant). Under these circumstances, research approaches that have been successful in identifying genes for Mendelian disorders are difficult and of limited power. The availability of new tools such as the development of PCR, the discovery of the class of DNA polymorphisms known as microsatellites and their systematic characterization as markers, the construction of genetic and physical maps of human genome, the development of non-parametric methods of analysis of genetic data and finally the completion of Human Genome Project, help us in finding the genes responsible for complex disorders, including type2 diabetes (Lander et al, 2001).



1.7.1. Monogenic Form of Diabetes

These are special types of familial diabetes that are transmitted with a mendalian mode of inheritance and share some clinical features with DM-2. Three main categories are identified they are

a) Maturity –onset diabetes of young (MODY): This is an autosomal dominant mode of inheritance. Impaired gene identified in MODY are

glucokinase (GCK-1), hepatic nuclear factor 1α &4α, insulin promoter factor 1, HNF1βand neuro D1 (Yamagata et al, 1996).

b) Genetic syndrome of extreme insulin resistance: It is rarer than MODY. Mutation in insulin-receptor gene, lamin A/C gene are identified (Shackleton et al, 2002).

c) Mitochondrial diabetes:- the maternally inherited diabetes and neurosensory deafness syndrome are caused by a single nucleotide mutation in the mtDNA (Reardon et al, 1992).

1.7.2. Multifactorial Forms of Type 2 Diabetes Mellitus

Two main approaches have been followed to identify genes for multi factorial forms of type 2 diabetes: a) studies of functional candidate genes and b) whole genome screens followed by the study of positional candidate genes.

Genes involved in insulin action: Mutation occurs to insulin receptor gene (INSR gene), insulin receptor substrates (IRS-1) and PI3-kinase, genes involved in glucose transport and metabolism and inhibitors of insulin actions, glycoprotein-1 and TNF-α.

Genes involved in insulin secretion: All the genes, whose products are involved in insulin secretion pathway are obvious candidate genesfortype 2 diabetes. Additional candidate genes are those involved in the development and turnover of β-cells or are part of hormonal system regulating β-cell activity. Mutations occur to GLUT2 and glucokinases, ion-channels, β-cell transcription factors and incretin cause type 2 diabetes mellitus.

Obesity genes: obesity is closely correlated to the development of type2 diabetes and also have some degree of heritability. Changes occur to some genes involved in central nervous system mediators, leptin and leptin receptors, β3-adrenergic receptors, and PPARγ may lead to diabetes mellitus (Doria 2010).

1.8. Definitions, Classification and Description of Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action.

Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia. There are many associated metabolic abnormalities—notably, the developmentof hyperketonaemia when there is a severe lack of insulin, together with alterations of fatty acids, lipids, and protein turnover. Diabetes is a permanent condition in all but a few special situations in which it can be transient.

1.8.1. Classification of diabetes mellitus and other categories of glucose regulation

1.8.1.1. Etiologic classification of diabetes mellitus (World Health Organization, 1985)

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)

A. Immune mediated; B. Idiopathic

II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance).

III. Other specific types:

A. Genetic defects of β-cell function: 1. Chromosome 12, HNF-1 (MODY3); 2. Chromosome 7, glucokinase (MODY2); 3. Chromosome 20, HNF-4 (MODY1); 4. Mitochondrial DNA;

B. Genetic defects in insulin action: 1. Type A insulin resistance; 2. Leprechaunism; 3. Rabson-Mendenhall syndrome; 4. Lipoatrophic diabetes;

C. Diseases of the exocrine pancreas: 1. Pancreatitis; 2. Trauma/pancreatectomy; 3. Neoplasia; 4. Cystic fibrosis; 5. Hemochromatosis; 6. Fibrocalculous pancreatopathy;

D. Endocrinopathies: 1. Acromegaly;2. Cushing's syndrome; 3. Glucagonoma; 4. Pheochromocytoma; 5. Hyperthyroidism; 6. Somatostatinoma; 7. Aldosteronoma;

E. Drug- or chemical-induced: 1. Vacor; 2. Pentamidine; 3. Nicotinic acid; 4.Glucocorticoids; 5. Thyroid hormone; 6. Diazoxide; 7. β-adrenergic agonists; 8. Thiazides; 9. Dilantin; 10. Interferon;

F. Infections:1. Congenital rubella; 2. Cytomegalovirus;

G. Uncommon forms of immune-mediated diabetes: 1. "Stiff-man" syndrome; 2. Anti-insulin receptor antibodies;

H. Other genetic syndromes sometimes sometimes associated with diabetes: 1. Down's syndrome;

2. Klinefelter's syndrome; 3. Turner's syndrome; 4. Wolfram's syndrome; 5. Friedreich's ataxia; 6. Huntington's chorea; 7. Laurence-Moon-Biedl syndrome; 8. Myotonic dystrophy; 9. Porphyria; 10. Prader-Willi syndrome;

IV. Gestational diabetes mellitus (GDM)

Criteria for the diagnosis of diabetes mellitus (National Diabetes Data Group, 1979; American Diabetes Association 2008).

1. Symptoms of diabetes plus casual plasma glucose concentration = 200 mg/dl (11.1 mmol/l). (Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss.)
2. Fasting Plasma Glucose = 126 mg/dl (7.0 mmol/l). (Fasting is defined as no caloric intake for at least 8 hour)

3. 2-hour Postprandial Glucose = 200 mg/dl (11.1 mmol/l) during an Oral Glucose Tolerance Test. The test should be performed as described by WHO (2), using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in 250 – 300 ml of water.
4. HbA1c as targets in a diabetic patient is 6.5-7.5% and as a screening test for type 2 diabetes mellitus is 6.0%.

1.9. Evaluations, Index Calculations and Oral glucose Tolerance Test in type 2 diabetes mellitus

1.9.1. Direct measures of insulin sensitivity

1.9.1.1. Hyperinsulinemic Euglycemic glucose clamp

The glucose clamp method is the reference standard for the direct determination metabolic insulin sensitivity in humans (De Fronzo et al, 1979). After an overnight fast, insulin is infused intravenously at a constant rate it the range of 5 to 120 mU/m^2/min (Units of insulin per body surface area per minute), resulting in a steady state insulin level that is above the fasting level (Hyperinsulinemic). As a result of this, glucose disposal in skeletal muscle and adipose tissue is increased and hepatic glucose production (HGP) is suppressed, decreasing blood glucose levels. A bedside glucose analyser is used to frequently monitor blood glucose levels at 5 - 10 minute intervals. Infusion of 20% dextrose is given intravenously at a variable rate to "clamp" blood glucose concentrations in the normal range (Euglycemic glucose clamp). Also, an infusion of potassium phosphate is given to prevent hypocalemia resulting from hyperinsulinemia and increased glucose disposal. Assuming that the artificially induced hyperinsulinemia state is sufficient to completely suppress HGP, and since there is no net change in blood glucose concentration under steady-state clamp conditions, the GIR (glucose infusion rate) must be equal to the glucose disposal rate (M). The whole body glucose disposal at a given level of hyperinsulinemia can be determined directly by this method. M is typically normalized to body weight or fat-free mass to generate

an estimate of insulin sensitivity. Alternatively, an insulin sensitivity index (S_I) derived from clamp data can be defined as $\text{SI clamp} = \frac{M}{(G \times \Delta I)}$, where M is normalized for G (steady state blood glucose concentration) and ΔI (difference between fasting and steady state plasma insulin concentrations) (Katz et al, 2000).

The validity of glucose clamp measurements of insulin sensitivity depends on achieving steady state conditions where glucose levels are fixed within the healthy reference range. Second important assumption of the glucose clamp method is that HGP is completely suppressed by steady-state hyperinsulinemia. The third critical consideration for interpretation of glucose clamp data is that M is routinely obtained only at a single insulin infusion rate. The fourth validity criteria is that the choice of this rate determines a level of hyperinsulinemia that is roughly comparable among all subjects studied. Importantly, the use of a single insulin infusion rate for comparisons of insulin sensitivity/resistance assumes that the steady-state insulin level achieved is in the range where M can vary according to differences in insulin sensitivity.

The main advantage of using the glucose clamp to estimate insulin sensitivity/resistance in human is that it directly measures whole body glucose disposal at a given level of insulinemia under steady-state conditions. The limitations of the glucose clamp approach are that it is time consuming, labor intensive, expensive and requires an experienced operator to manage the technical aspects.

1.9.1.2. Insulin suppression test

After an overnight fast, somatostatin (250 µg/h) or the somatostatin analog octreotide (25 µg bolus, followed by 0.5 µg/min) (Pei et al, 1994) is intravenously infused to suppress endogenous secretion of insulin and glucagon. Simultaneously, insulin (25 mU/$m^{2/}$min) and glucose (240 mg/$m^{2/}$min) are infused into the same ante cubital vein for three hours. From the other arm, blood sample for glucose and insulin determination are taken every 30 minute for 2.5 hours and then at 10 minutes intervals from 150 to 180 minute of the Insulin Suppression Test. The constant infusion of insulin and glucose will determine steady-state plasma insulin (SSPI) and glucose (SSPG) concentration. The steady-state period is assumed to be

from 150 - 180 minute after initiation of the Insulin suppression test. SSPI concentrations are generally similar among subjects. SSPG values are inversely related to insulin sensitivity. The IST provides a direct measure (SSPG) of the ability of exogenous insulin to mediate disposal of an intravenous glucose load under steady-state conditions where endogenous insulin secretion is suppressed.

As with glucose clamp, the validity of IST depends on achieving steady state conditions. The SSPG is a highly reproducible direct measurement of metabolic actions of insulin that is less labor intensive and less technically demanding than the glucose clamp.

1.9.2. Indirect measures of Insulin Sensitivity

1.9.2.1. Minimal model analysis of frequently sampled intravenous glucose tolerance test

The minimal model described by Bergman et al, 1976, provide an indirect measurement of metabolic insulin sensitivity/ resistance on the basis of glucose and insulin data obtained during a frequently sampled intravenous glucose tolerance test (FSIVGTT). After an overnight fast, an intravenous bolus of glucose (0.3 g/kg body weight) is infused in two minutes starting at time zero. Currently, a modified FSIVGTT is used where exogenous insulin (4 mU/kg/minute) is also infused within a time duration of 5 minutes, beginning at 20 minutes after the intravenous glucose bolus. Blood samples are taken for plasma glucoseand insulin measurements at -10, -1, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 160 and 180 minutes. These data are then subjected to minimal model analysis using the computer program MINMOD to generate an index of insulin sensitivity (S_I).

Minimal model uniquely identifies model parameter that determines a best fit to glucose disappearance during the modified FSIVGTT. S_I: fractional glucose disappearance per insulin concentration unit; S_G: ability of glucose per se to promote its own disposal and inhibit HGP in the absence of an incremental insulin effect (i.e. when insulin is at basal level).

1.9.2.2. Oral glucose tolerance test/meal tolerance test

The oral glucose tolerance test (OGTT) is a simple test widely used in clinical practice to diagnose glucose intolerance and type 2 diabetes (American Diabetes Association, 2007). After overnight fast, blood samples for determination of glucose and insulin concentration are taken at 0, 30, 60, and 120min following a standard oral glucose load (75 g) or a standard meal (Dalla Man et al, 2005). Oral glucose tolerance reflects the efficiency of the body to dispose of glucose after oral glucose load or meal. It is important to recognize that glucose tolerance and insulin sensitivity are not equivalent concepts. The OGTT and meal tolerance test provide useful information about glucose tolerance but not insulin sensitivity/ resistance per se.

1.9.2.3. Simple Surrogate Index for Insulin Sensitivity/ Resistance

Surrogates derived from fasting steady state condition: After an overnight fast, a single blood sample is taken for determination of blood glucose and plasma insulin. In healthy humans, the fasting condition represents a basal steady state where glucose is homeostatically maintained in the normal range such that insulin levels are not significantly changing and HGP is constant; i.e. basal insulin secretion by pancreatic β-cells determines a relatively constant level of insulinemia that will be lower or higher in accordance with insulin sensitivity/ resistance such that HGP matches whole body glucose disposal under fasting condition

Surrogate indexes based on fasting glucose and insulin concentrations reflect primarily hepatic insulin sensitivity/resistance. However under most conditions, hepatic and skeletal muscle insulin sensitivity/resistance is proportional to each other. In the diabetic state with fasting hyperglycemia, fasting insulin levels are inappropriately low and insufficient to maintain euglycemia.

I/ (fasting insulin): In healthy subjects, elevations in fasting insulin levels (with normal fasting glucose level) correspond to increased insulin resistance. Indeed, in nondiabetic subjects, I/(fasting insulin) is a well-known proxy for insulin sensitivity that decreases as subjects become more insulin resistant (and fasting insulin levels rise) (Laakso, 1993) This index does not take into account the inappropriately low insulin secretion in the phase of hyperglycemia seen in diabetic subjects or glucose intolerant subjects. Consequently, using I/ (fasting insulin) as a measurement of insulin sensitivity/resistance in patients with glucose

intolerance or type 2 diabetes who have diminished pancreatic reserve leads to erroneous results.

Glucose/insulin ratio: A number of studies have used the fasting glucose/insulin ratio (G/I ratio) as an index of insulin resistance. In case of non diabetic subjects, the G/I ratio is essentially functionally equivalent to I/ (fasting insulin) since fasting glucose levels are all in the normal range. The G/I ratio paradoxically and erroneously increases in the diabetic subjects. Therefore the fasting G/I ratio is a conceptually flawed index of insulin sensitivity. (Vuguin & Saenger, 2001)

Homeostasis model assessment: Homeostasis model assessment (HOMA), (Matthews et al, 1985), is a model of interactions between glucose and insulin dynamics that is then used to predict fasting steady state glucose and insulin concentrations for a wide range of possible combinations of insulin resistance and β-cell function. Both the original HOMA and the updated HOMA too assume a feedback loop between the liver and β-cell. The model predicts fasting steady state levels of plasma glucose and insulin for any given combination of pancreatic β-cell function and insulin sensitivity. In practical terms, most studies using HOMA employ an approximation describe by a simple equation to determine a surrogate index of insulin resistance. This is defined by the product of the fasting glucose and fasting insulin divided by a constant.

Homeostasis Model Assessment of Insulin Resistance, HOMA-IR = {[fasting insulin (μU/ml)] X [fasting glucose (mmol/l)]}/22.5. The denominator of 22.5 is a normalizing factor; i.e. the product of normal fasting plasma insulin of 5 μU/ml and normal fasting plasma glucose of 4.5 mmol/l typical of a "normal" healthy individual equal to 22.5. Therefore, for an individual with "normal" insulin sensitivity, HOMA-IS = 1/HOMA-IR, has a reasonable linear correlation with glucose clamp and minimal model estimates of insulin sensitivity/resistance in several studies of distinct populations. The coefficient of variation for HOMA-IR varies considerably depending upon the number of fasting samples obtained and the type of insulin assay used (Wallace TM, et al. 2004). It is important to note that, over wide ranges of insulin sensitivity/ resistance, log (HOMA-IR) transforms skewed distribution of fasting insulin values to determine a much stronger linear correlation with glucose clamp estimates of insulin sensitivity (Katz A, et al,

2000). Log (HOMA-IR) is useful for evaluation of insulin resistance in individuals with glucose intolerance, mild to moderate diabetes and other insulin resistant conditions. However, in subjects with severely impaired or absent β-cell function, HOMA-IR may not give appropriate results. HOMA or log (HOMA) is used extensively in large epidemiological studies, prospective clinical trials, and clinical research studies.

Quantitative insulin sensitivity check index (QUICKI): QUICKI is an empirically derived mathematical transformation of fasting blood glucose and plasma insulin concentrations that provide a reliable, reproducible, and accurate index of insulin sensitivity with excellent positive predictive power (Chen et al, 2005). During development of QUICKI, sensitivity analysis of data from the first 20 minute of an FSIVGTT revealed that physiological fasting steady state values of plasma insulin and glucose contain critical information about insulin sensitivity as determined by the reference standard glucose clamp(SI_{clamp}). Since fasting insulin level have a non normal skewed distribution, log transformation improves its linear correlation with $SI_{clamp.}$ However, as with 1/ (fasting insulin) and the G/I ratio, this correlation is not maintained in diabetic subjects with fasting hyperglycemia and impaired β-cell function that is insufficient to maintain euglycemia. QUICKI=1/ [log (fasting insulin, μU/ml) + log (fasting glucose, mg/dl)]. Over a wide range of insulin sensitivity/resistance QUICKI has a substantially better linear correlation with SI_{clamp}($r \approx 0.8$-0.9) than S_I derived from the minimal model or HOMA-IR. QUICKI is proportional to 1/log (HOMA-IR). QUICKI perform best in insulin resistant subjects, whereas S_I from the minimal model performs best in healthy, insulin sensitive subjects. Test characteristics examining repeatability and reliability of QUICKI (including coefficient of variation and discriminant ratio) demonstrate that QUICKI is superior to 1/ (fasting insulin), log (insulin) and HOMA-IR and comparable with log (HOMA) and GIR from the glucose clamp. QUICKI is among the most thoroughly evaluated and validated surrogate index for insulin sensitivity.

Surrogates Derived from Dynamic Tests

Specific indexes including Matsuda index, Stumvoll Index, Avignon index, Oral glucose insulin sensitivity index, Gutt Index and Belfiore index use

particular sampling protocols during the OGTT or the meal. Glucose disposal after an oral glucose load or a meal is mediated by a complex dynamic process that includes absorption, glucose effectiveness, neuro-hormonal actions, incretin action, insulin secretion and metabolic actions of insulin that primarily determine the balance between peripheral glucose utilization and HGP. Surrogate indexes that depend on dynamic testing take into account both fasting steady state and dynamic post glucose load plasma glucose and insulin levels. Surrogates derived from dynamic testing generally incorporate both peripheral and hepatic insulin sensitivity. The advantage of surrogates based on dynamic testing is that information about insulin secretion can be obtained at the same time as information about insulin action.

Insulin sensitivity index-Matsuda (Matsuda & De Fronza, 1999): Insulin sensitivity index-Matsuda ($ISI_{Matsuda}$) is an insulin sensitivity index that reflects a composite estimate of hepatic and muscle insulin sensitivity determined from OGTT data. $ISI_{Matsuda}=10{,}000/\sqrt{[(G_{fasting} \times I_{fasting}) \times (G_{OGTT\ mean} \times I_{OGTT\ mean})]}$, where fasting glucose and insulin data are taken from time zero of the OGTT and man data represent the average glucose and insulin values obtained during the entire OGTT. The square root is used to correct for non linear distribution of insulin and 10,000 is a scaling factor in the equation. $ISI_{Matsuda}$ correlates reasonably well with estimates of whole body insulin sensitivity determined by the glucose clamp. The fasting component reflects hepatic insulin sensitivity, whereas the mean of the dynamic data primarily represents skeletal muscle insulin sensitivity. The insulin secretion/insulin resistance (disposition) index calculated as the product of insulin secretion measured with ($\frac{\Delta I_{0-30}}{\Delta G_{0-30}}$ or $\frac{\Delta I_{0-120}}{\Delta G_{0-120}}$) and $ISI_{(Matsuda)}$ (or modified $ISI_{Matsuda}$ using plasma glucose and insulin concentration at 30 minute during the OGTT) had excellent power to predict onset of Type 2 diabetes.

1.10. Reference values: Establishment and uses

An objective of this dissertation is to calculate the reference interval of a clinical parameter, understand its clinical nature, its baseline character and partition the reference interval, if required. So a brief review about reference interval and its method of calculation is explained below.

1.10.1. Establishment of reference values

From clinical interviews and history, clinical examination and supplementary investigations, the collected data must be interpreted by comparison with reference data to make inferences of the presence or absence of a disease. Interpretation of medical laboratory data is a case of decision making by comparison. Reference values are required for all clinical laboratory tests performed, not only from healthy individuals but also from patients with relevant diseases. Some conditions are very important to make the comparison of patient's laboratory results with reference values possible and valid (Solberg & Grasbeck, 1989):

1. All groups of reference individuals should be clearly defined.
2. Conditions in which the sample were obtained and processed for analysis should be known.
3. All quantities compared should be of same type.
4. All laboratory results should be produced with the use of adequately standardized methods under sufficient analytical quality control.
5. The diagnostic sensitivity and specificity, prevalence and clinical costs of misclassification should be known for all lab tests used.

Reference interval is a certain type of quantity obtained from a single individual or group of individuals corresponding to a stated description which must be spelled out and made available for use by others also or in other words reference value is a value obtained by observation or measurement of a particular type of quantity on a reference individual. Reference individual is an individual selected, as basis for comparison with individuals under clinical investigation, through the use of defined criteria. Reference value can be divided into two–subject based and population based. Subject based reference values are previous values from the same individual obtained when the individual was in a defined healthy state. Population based reference values are those obtained from a group of well defined reference individuals.

1.10.2. Selection of the Reference individual

Individuals, who fulfill a set of selection criteria, are included in the reference group. The source population specification of criteria for health, the disease of interest etc. are some selection criteria. Some of the exclusion criteria for health-associated reference values are as follows: a) Diseases; b) Risk factors – obesity, hypertension, risk from occupation or environment, genetically determined risks; c) Intake of pharmacologically active agents, drug treatment for disease or suffering, oral contraceptives, drug abuse; d) Alcohol, tobacco; e) Specific physiological states – pregnancy, stress, excessive exercise.

From the parent population, who fulfill the selection criteria, random sampling is the best method for selecting reference individuals. But strict random sampling is not possible due to practical reasons. Therefore using the best reference sample that can possibly be obtained after all practical considerations have been taken into account is necessary (Horowitz, 2012).

1.10.3. Partitioning of the Reference Data

It is necessary to have separate reference values for sex, age group and other criteria. The number of partitions criteria should be kept small, so that sufficient sample sizes for valid statistical estimate must be obtained. Several analytes vary significantly among different age and gender groups, so age and sex are most frequently used criteria for sub grouping. Some examples of partition criteria to be used for possible sub grouping are as follows: a) Age; b) Sex; c) Genetic factors (race, blood groups, HLA); d) Physiological factors (stage of menstrual cycle, pregnancy, physical condition); and e) other factors (socio economic, environmental & chronobiological).

1.10.4. Sample collection for the Reference Data

The next step is blood sample collection. To eliminate or minimize bias or variation, standardization of preanalytical preparation of individual before sample collection, sample collection itself and handling of sample before analysis etc. are necessary. Venipuncture and skin puncture are standardised procedures for blood sample collection (Grasbeck & Alstrom, 1981). Same analytical method should be used to ensure comparability between reference and observed value. Reduction of preanalytical variables such as diurnal variations, food intake, stress should be kept

to a minimum by defining the time of sample collection. Sample preparation and storage also affects the reference data.

1.10.5. Analytical Procedure

After sample collection its analysis is very important. In the analytical procedures the essential components required specification are: a) Analysis method, including information on equipment, reagents, calibration type of raw data and calculation methods, b) quality control, c) reliability, reproducibility and validity criteria for the data. The specification should be so clear that another investigator can reproduce the study.

1.10.6. Statistical Treatment

The reference values are subjected to statistical treatment after the analysis of specimen. This includes partitioning of reference values into appropriate groups, inspection of distribution of each group, identification of outliers and determination of reference limits (Sachs, 1984).

The reference values may be partitioned according to sex, age and other characteristics. Partitioning is also known as stratification, categorization or sub grouping and its results are known as partitions, strata, categories, classes or subgroups. The aim of partitioning is to reduce variations among subjects to minimize biological "noise". Less intraclass variations gives narrower and more sensitive reference intervals. When the difference between the classes is statistically significant, the reference values may be partitioned (Shultz, 1985).

A histogram must be prepared from the reference distribution. It will provide valuable information about the data and is a safeguard against the misapplication and misinterpretation of statistical method. The examination of distribution gives the following characteristic

a) Highly deviating values (outliers) may represent erroneous values.

b) Bimodal or polymodal distribution have more than one peak and may indicate that the distribution is non-homogeneous

c) The shape of distribution may be asymmetrical (skewed) or symmetrical.

d) The location of reference limits can be estimate by visual inspection of the distribution.

For the identification of possible outliers, visual inspection of a histogram is a reliable method. In a positively skewed distribution, the identification of outliers may aid by the logarithmic values of the distribution. Statistical tests are also used for outlier identification. The possible outliers should not be discarded automatically; it should be included or excluded on a rational basis. If some non-correctable causes found, then reject the deviated values (Horowitz, 2012).

1.10.7. Determination of Reference Limits

Reference interval, bounded by a pair of reference limits, is a useful information carried by the total set of reference values. Three kinds of reference intervals may described. They are tolerance interval, prediction interval and inter percentile interval. IFCC recommend the inter percentile interval, which is simple to estimate and commonly used. It is defined as an interval bounded by two percentiles of reference distribution. A percentile denotes a value that divides the reference distribution into specified percentage, which has magnitudes less than or equal to the limiting value. The reference interval as the central 95% interval bounded by 2.5 and 97.5 percentile is common but is arbitrary: that is, 2.5% of values are cut-off in both tails of reference distribution. It is a non parametric method and is not dependent of the Gaussian distribution of the sample. The precision of the percentile depends on the subset size, if a few observations are present, it is less precise. The determination of the confidence interval of the percentile is possible when the random sampling is fulfilled. Theoretically, 40 values are required in a minimum sample size for the estimation of the 2.5 and 97.5 percentile, but at least 120 reference values are required for reliable estimates.

Parametric and non-parametric statistical techniques are used for the determination of the inter percentile interval. The Parametric method is based on the estimates of population parameters, such as mean and standard deviation (SD). The reference distribution must be Gaussian distribution or must be transformed to approximately Gaussian distribution, and then only parametric technique works. Transformations may also be done to increase the statistic of the Gaussian distribution. In the parametric method, the reference limits are located 2 SDs below

and above the mean. The type of distribution is not important for non-parametric method. The percentiles are determined by simply cutting off the required percentage from each tail of the subset reference distribution. When the results obtained by these two methods compared, the percentiles estimated are very similar; the only difference is that the parametric method is theoretically more precise. The simple and reliable non-parametric method with bootstrap is generally preferred than parametric method.

The uses of reference values include presentation of an observed value, also are used in multivariate reference region, subject based reference values, determination of the transferability of reference values and in sensitivity and specificity. An observed value is compared with reference values. These observed values may be classified as low, usual or high depending on its location in relation to the reference interval. The multivariate analysis helps to prevent the false-positive results. A multivariate distribution is a cluster of points on a plane, in a space or in a hyperspace, depending on the dimensionality of the observation. The multivariate reference region can be defined based on the joint distribution of the reference values for two or more laboratory tests. A computer program is needed for the use of multivariate reference region. The application of subject based reference values becomes more feasible as health screening by laboratory tests and computer storage of results become available to large section of the general population.

It is convenient to use the reference values in one facility that is generated in another laboratory. This is possible when some conditions are fulfilled such as the population must be described and matched adequately, the data should be checked for bias arising from analytical factors, individual preparation before specimen collection and specimen collection itself should follow standardized scheme in both laboratory. For the correct interpretation of a result, the sensitivity and specificity of a test must be known. The sensitivity means the fraction of subjects with a specific disease that assay correctly predicts. Specificity correctly predicts those individuals without the disease. Changing the decision limits of an assay affects both sensitivity and specificity.

1.11. Assessment various diagnostic criteria and risk of a disease

1.11.1. Sensitivity and specificity

Sensitivity is the proportion of positives that are correctly identified by the test. The sensitivity of a test is its true-positive rate, and is expressed as the ratio of true-positive results to all of those with the disease [true positive/ (true positive + false negative)]. Specificity is the proportion of negatives that are correctly identified by the test. The specificity of a test is its true-negative rate, and is expressed as the ratio of true-negative results to all of those without disease [true negative/ (true negative + false positive)]. The positive and negative refer to the presence or absence of the condition of interest. Test with high sensitivity are often used as screening tests as they help to identify true diseases. Tests with high specificity are used as confirmatory tests and are some-times applied to a group identified by a screening test in order to decrease the number of false-positive results. The disadvantage of the sensitivity and specificity is that they do not assess the accuracy of the test in a clinically useful way (Riffenburgh, 2012).

1.11.2. Positive and negative predictive value

The whole point of a diagnostic test is to use it to make a diagnosis, so we need to know what the probability is of the test giving the correct diagnosis, whether it is positive or negative. The sensitivity and specificity do not give us this information. Instead we must approach the data from the direction of the test results. Positive predictive value is the proportion of patients with positive test results who are correctly diagnosed; negative predictive value is the proportion of patients with negative test results who are correctly diagnosed. The positive and negative predictive values give a direct assessment of the usefulness of the test in practice.

1.11.3. The likelihood ratio

For any test result we can compare the probability of getting that result if the patient truly had the condition of interest, with the corresponding probability if they were healthy. The ratio of these probabilities is called the likelihood (LR), and it is calculated as

$$\mathrm{LR} = \frac{prob(positive test/\mathrm{disease})}{prob(positive\ \mathrm{test/no\ disease})} = \frac{sensitivity}{1-specificity}$$

We can consider the likelihood ratio as indicating the value of the test for increasing certainty about a positive diagnosis. The prevalence is the probability of disease before the test is performed. The *odds* of having the disease are thus given as prevalence/ (1-prevalence). Thus if the prevalence is 10%, the odds are 0.11, or 9 to 1 against the disease being present. We can call this figure the pre-test odds, and the odds corresponding to the positive predictive value as the post-test odds.

Post-test odds = pre-test odds xlikelihood ratio

A high likelihood ratio may demonstrate that the test is useful but it does not necessarily indicate that a positive test is a good indicator of the presence of disease. Using odds rather than probabilities may be helpful, however, especially for seeing the usefulness of the test as assessed by the likelihood ratio (Riffenburgh, 2012).

1.11.4. ROC curve

When a measurement is used to make a diagnosis the choice of the 'best' cut-off is not simple. A graphical approach is to plot the sensitivity versus 1-specificity for each possible cut-off, and to join the points. The curve thus obtained is known as a 'receiver operating characteristic' curve or ROC curve, because the method originated in studies of signal detection by radar operators.

The ROC method is perhaps most useful when comparing two or more competing methods. For a single test it does not add anything to a table but it is preferable when there are many possible cut-off values. Of course, the ROC curve, being based only on sensitivity and specificity, takes no account of the prevalence of the disease being tested for.

1.11.5. Odds ratio

There is yet another way of analyzing two by two tables, which involves the comparison of two groups with respect to the risk of some events. The best way to compare methods of delivery is to use odds ratios. The odds ratio (OR) gives the odds of disease when predicted in ratio to the odds of disease when not predicted, indicating the usefulness of the prediction method. The OR gives us some indication of level of association between the two variables. The odds ratio offers a

method of getting an approximate relative risk despite the method of sample selection.

An odds ratio is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure. Odds ratios are most commonly used in case-control studies; however they can also be used in cross-sectional and cohort study designs as well.

Odds ratios are used to compare the relative odds of the occurrence of the outcome of interest (e.g. diseases or disorders), given exposure to the variable of interest (e.g. health characteristic, aspect of medical history) the odds ratio can also be used to determine whether a particular exposure is a risk factor for a particular outcome, and to compare the magnitude of various risk factors for that outcome.

OR = 1, exposure does not affect odds of outcome

OR > 1, exposure associated with higher odds of outcome

OR < 1, exposure associated with lower odds of outcome

The 95% confidence interval (CI) is used to estimate the precision of the OR. A large CI indicates low level of precision of the OR, whereas a small CI indicates a higher precision of the OR. It is important to note however, that unlike the p value, the 95% CI does not report a measure's statistical significance. In practice the 95% CI is often used as a proxy for the presence of statistical significance if it does not overlap the null value (e.g. OR = 1). Nevertheless, it would be inappropriate to interpret an OR with 95% CI that spans the null value as indicating evidence for lack of association between the exposure and outcome.

When a non-casual association is observed between a given exposure and outcome is as a result of the influence of a third variable, it is termed confounding, the third variable is termed a confounding variable. A confounding variable is casually associated with the outcome of interest, and non-casually or casually associated with the exposure, but is not an intermediate variable in the casual pathway between exposure and outcome. Stratification and multiple regression

technique are two methods used to address confounding, and produce "adjusted" OR's (Szumilas, 2010.

	Outcome status		
Exposure status		+	-
	+	A	B
	-	C	D

Where A = Number of exposed cases; B = Number of exposed non-case; C = Number of unexposed cases; D = Number of unexposed non-cases

$$OR = \frac{a/c}{b/d} = \frac{ad}{bc}$$

$$\frac{(n)exposedcases/(n)unexposedcases}{(n)exposednon-cases/(n)unexposednon-cases} = \frac{(n)exposedcasesX(n)unexposednon-cases}{(n)exposednon-casesX(n)unexposedcases}$$

1.12. Gender Differences in Type 2 Diabetes Mellitus

1.12.1. Estrogen action, Receptor, hypothalamus and Energy Balance

There isdecrease in circulating estrogens, as women enter menopause. Entry into menopause is associated with alterations in energy homeostasis, resulting in increase in intra abdominal body fat (Carr, 2003). In animals, ovariectomy leads to increased adiposity (Wallen et al, 2001). Although overiectomy induces a transient increase in food intake in rodents and E2 replacement decreases food intake (Wallen et al, 2001). Reduced E2 synthesis resulting from aromatase inactivation promotes obesity in the absence of hyperphagia or reduced energy expenditure in mice of both sexes. The animals exhibit reduced spontaneous physical activity and lean body mass (Jones et al, 2000). ER alpha deficiency in male and female mice caused increase in body weight and adiposity through reduced energy expenditure and slight increases in

food intake (Heine et al, 2000). Although endogenous E2 favors body weight homeostasis by increasing energy expenditure (Rogers et al, 2009), exogenous estrogens may promote energy balance by influencing both energy intake and energy expenditure. Thus, global loss of ER alpha action results in a decrease in energy expenditure, whereas increased ER alpha (and ER beta) signaling resulting from elevating serum E2 concentrations suppresses energy intake and increases energy expenditure.

Hypothalamus in the central nervous system controls food intake, energy expenditure and body weight homeostasis. Dramatic changes in all of these characteristics can be induced by lesioning specific hypothalamic nuclei, such as the ventromedial hypothalamus (VMH) (Smith, 2000) or the lateral hypothalamic area (Danguir & Nicolaidis, 1980; Milam et al, 1980). ER alpha is abundantly expressed in the rodent brain in the ventrolateral portion of the ventromedial nucleus (VMN), the arcuate nucleus (ARC), the medial preoptic area, and the paraventricular nuclei (Simerly, 1990; Simonian & Herbison, 1997; Voisin et al, 1997). ER beta is found in the same hypothalamic nuclei, but ER beta expression is significantly lower relative to ER alpha.

Pharmacological studies showing that, whereas the selective ER alpha agonist propylpyrazole triol (PPT) suppresses food intake in ovariectomized mice, the selective ER beta agonist diarylpropionitrile failed to influence feeding behavior at any dose (Roesch, 2006; Santollo et al, 2007). E2 also suppresses food intake through ER beta because the anorectic effect of intracerebroventricular injection of E2 is blocked by co administration of ER beta antisense oligodeoxynucleotides in female rats.

1.12.1.1. Estrogen and Regulation of Adipose Tissue Distribution

There is a sexual dimorphisms described for body fat distribution. Men, on average, have less total body fat but more central/intra-abdominal adipose tissue, whereas women tend to have more total fat that favors gluteal and femoral and subcutaneous depots (Bouchard et al, 1993; Enzi et al, 1986 & Bjorntorp, 1992). After menopause, fat distribution shifts in women and the distribution changed to a pattern more similar to that of men (Guthrie et al, 2004 & Lovejoy et al, 2008). But

estrogen replacement therapy prevents the male-patterned accumulation of intra-abdominal adipose tissue (Gambacciani et al, 1997).

1.12.1.2. Intra-abdominal adipose tissue and themetabolic syndrome

Excess accumulation of adipose tissue in the central region of the body, otherwise known as the intra abdominal or male pattern obesity (Wajchenberg, 2000) correlates with increased risk of, and mortality from, disorders including type 2 diabetes mellitus, hyperlipidemia, hypertension and atherosclerosis. Intra abdominal adipose tissue is thought to be metabolically and functionally different from subcutaneous adipose tissue. Intra abdominal adipose tissue has more capillaries and efferent sympathetic axons per unit volume than subcutaneous fat, and unlike subcutaneous fat, it drains into the hepatic portal vein (Wajchenberg. 2000). In humans, surgical removal of intra abdominal adipose tissue by omentectomy during bariatric surgery has been shown to either decrease insulin and glucose levels (Thorne et al, 2002) or to have no effect on components of the metabolic syndrome and insulin resistance (Herrera et al, 2010, Fabbrini et al, 2010).

1.12.1.3. Subcutaneous adipose tissue and lipid storage

Subcutaneous adipose tissue deposition is not associated with the metabolic disturbances due to its enhanced ability to expand. This allows for storage of excess caloric intake through angiogenesis and reduced hypoxia and fibrosis (Wajchenberg, 2000). Females have more subcutaneous adipose tissue than males. Subcutaneous adipose tissue is dispersed in a broad area under the skin, is relatively poorly innervated and vascularized, and has a larger average cell diameter than intra-abdominal adipose tissue (Wajchenberg, 2000). Subcutaneous adipose tissue permits efficient storage of maximal calories per unit volume of tissue. Lipid deposition into subcutaneous adipose tissue may provide an evolutionary advantage for females because it provides protection from fluctuations in caloric supply, thereby maintaining reproductive capacity. Females also mobilize adipose tissue stored in the subcutaneous depot to augment the caloric demands associated with breast feeding.

1.12.2. Estrogen and Insulin Sensitivity

Insulin resistance is central to the progress towards type 2 diabetes mellitus and in established diabetes. It is also central to the development of metabolic syndrome. Compared with age-matched men, women with a normal menstrual history have enhanced insulin sensitivity normalized to lean mass. This phenomenon contributes to the reduced incidence of type 2 diabetes observed in premenopausal women (Park et al, 2003; Yki-Jarvinen H, 1984). E2-replete females, humans and rodents, are typically protected against a high fat diet - and acute fatty acid-induced insulin resistance (Frias et al, 2001; Djouadi et al, 1998; Hong et al, 2009). After menopause or overectomy, a sudden decline in insulin sensitivity parallels an increase in fat mass and elevations in circulating inflammatory markers, low-density lipoprotein (LDL), triglycerides and fatty acids (Carr, 2003; Pfeilschifter et al, 2002; Sites et al, 2002).

1.13. Biostatistics

Biostatistical methods used in this study are defined and described below (Reffenburg, 2012; Gurumani, 2005; Altman, 1991).

1.13.1. Mean: Arithmetic mean, commonly called mean, is the sum of all the variants of a variable divided by the total number of items in the samples. It is types of average.

$$\text{Mean} = \frac{sumofall\ t\square eitemsinasample}{Totalnumberofitemsint\square esample}$$

1.13.2. Median: Median is the value of the middle items of a given series of data arranged in ascending order of the magnitude, i.e., in an array.

$$\text{Median} = \frac{valueoftheitem\ (n+1)}{2} \text{ in an array}$$

1.13.3. Mode: Mode is defined as that value which occurs most frequently in a sample.

1.13.4. Standard Deviation (SD): It is defined as the square root of the arithmetic mean of the squared deviation of various items from arithmetic mean. In short, it is called the root-mean-square-deviation. The mean of squared deviations is called the variance. Therefore, the square root of *variance* is the SD.

$$SD = \sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2}$$

Where,

X = variable X

$\bar{X}$ = mean of variable X = ΣX/n

$X - \bar{X}$ = deviation

$(X - \bar{X})^2$ = squared deviation

$(X - \bar{X})^2/n$ = mean of squared deviation = Variance

1.13.5. Range: It is a very simple measure of dispersion. Range is defined as the difference between maximum value and the minimum value of the given series of data (Gurumani, 2005).

1.13.6. 95% confidence interval of mean: Confidence interval is a range of values which we can be confident including the true values. A Confidence interval for the estimated mean extends either sides of the mean by a multiple of the standard error. 95% confidence interval was calculated by multiplying standard error by 1.96 and then identifying the range by adding and subtracting the value from mean.

1.13.7. Coefficient of Variation (CV): CV isalso known as relative standard deviation (RSD). It is a measure of dispersion of a probability distribution or frequency distribution. CV is defined as the ratio of standard deviation to the mean and is expressed in percentage. It is widely used in analytical chemistry to express the precision and repeatability of an assay.

1.13.8. Shapiro-Wilk Method for Tests of Normality: The Shapiro-Wilk test is a test of normality in frequentist statistics, published in 1965 by Samuel Sanford Shapiro and Martin Wilk. It utilizes the null hypothesis principle to check whether a sample x_1.....x_2 came from a normally distributed population.

1.13.9. Skewness: Skewness is a measure of symmetry, or more precisely the lack of symmetry. A distribution, or data set, is symmetric if it looks the same to the left and right of the center point. The skewness for a normal

distribution is zero and any symmetric data should have skewness near zero.

Negative values for the skewness indicate data that are skewed left and positive values for the skewness indicate data that are skewed right. The properties of positive skewed distribution is arithmetic mean > median > mode. The frequency curve has a steep rise and a slow fall with a long tail on the right.

The properties of negative skewed distribution is arithmetic mean < median < mode. The frequency curve has a slow rise and a deep fall with a long tail on the left.

By these properties we can tests the skewness and it showed as: frequency curve is not perfectly bell shaped; it is tailed either on the right (positive skewness) or on the left (negative skewness). Numerical measure developed to evaluate the skewness of a distribution is Karl Pearson's coefficient of skewness.

Karl Pearson's coefficient of skewness = $\frac{(Mean-Mode)}{Standarddeviation}$ = (-1 to +1).

1.13.10. Kurtosis: Measure the flatness of a frequency of items (classes) near the value of the mode, relative to a normal curve.

Coefficient of Kurtosis, $\beta_2 = \mu_4/\sigma^4$,

Where $\mu_4 = \Sigma(X - \overline{X})^4/n$ and

$\sigma^4 = \{\Sigma(X - \overline{X})^2/n\}^2$.

If $\beta_2 = 3$, the frequency curve is the mesokurtic (normal bell shaped curve).

if$\beta_2 < 3$, the frequency curve is platykurtic (flat),

if$\beta_2 > 3$ the frequency curve is leptokurtic (peaked) (Gurumani, 2005).

Bar Diagram: A method of presenting data in which frequencies are displayed along one axis and categories of the variable along the other, the frequencies being represented by the bar lengths.

1.13.11. Histogram: Histogram is the graphical representation of continuous frequency distribution. The X-axis has the true class intervals, and the Y-axis, the frequencies. The bars are of equal width indicating that the class-

intervals are of equal width. The height of the bar is proportional to the respective frequency. Therefore, it may be said that the area (length ×breadth) of each bar is equal to the total of all the frequencies.

1.13.12. Box-Whisker plot: Box-Whisker Plot is a graphical representation of data that gives visual information about measures of central tendency, distribution and information about outliers. A Box-Whisker Plot has a central reference scale on which the data points of the continuous data present is plotted. On this scale, the position of median, first quartile Q1, third quartile Q3, inter quartile range are calculated and plotted. The inner fence points on either side of the median are marked as f1 and f3 are calculated as follows

f1 = Q1 − 1.5 × Inter quartile Range

f3 = Q3 + 1.5 × Inter quartile Range

Adjacent value a1 is the value closest to f1 but not less than f1 and adjacent value a3 value is nearest to f3 but not above it. The outer fence f1 and f3 are calculated by the following equations

F1 = Q1 − 2 (1.5) (Inter quartile Range)

F3 = Q3 + 2 (1.5) (Inter quartile Range)

The box ending at Q1 and Q3 are drawn. A line cutting the box at the median is also drawn in the box. Adjacent value a1 and a3 are indicated and connected to the box by a dash line. Any value between f1 and F1 are the mild negative outliers and one between the inner and outer fence are indicated by a data point. These are mild negative outliers. Any value between f3 and F3 are the mild positive outliers. Values of the data that lie beyond the outer fences (f1 and F3) are the external outliers.

Interpretation of Box-Whisker plot: If the distribution is symmetrical, the median is in the middle of the box. If the median is towards the lower value point, away from the center of the box, then it is positively skewed. Similarly if the median point is deviated from the center to a higher value point, then it is negatively skewed.

The inner and outer fences are used to locate the mild and the extreme outliers. The outliers are samples outside the normal distribution scale. Outliers are identified and located for analysis of disease conditions or abnormal values or errors. Outliers may be removed in one or several steps to identify the normal distribution of the sample or transform a sample to normal distribution.

Centiles: The value, which is below a certain percentage, is called a centile or percentile. This value corresponds to a value with a particular cumulative frequency. For example; if we require 5^{th} and 95^{th} centiles of a distribution, we may arrange the total sample in ascending order. If the total number of sample is 'N', then the 5^{th} and 95^{th} centiles are identified by the following equation.

5^{th} centile = $0.05 \times N$

95^{th} centile = $0.95 \times N$

If the centile obtained is an integer, then that value is considered as the 5^{th} and 95^{th} centiles. But if the value calculated is between two numbers, and is a non-integer, then the following calculations are done. The adjacent two data points are taken and the following calculations are made.

Lower value + the centile fraction (higher value−lower value) = the particular centile value.

The 25^{th} and 75^{th} centiles are known as **quartiles**; these values together with the median divide the data into four equally populated subgroups. The numerical difference between the 25^{th} and 75^{th} centiles is the **inter-quartile range**, and is occasionally used to describe variability.

1.13.13. Normal quantile-quantile plot (Q-Q plot): In this method, a plot of the quantiles of the data distribution is plotted against the expected quantiles of the normal distribution. The expected quantiles of the normal distribution is expressed as ± z value of normal distribution. If the sample quantiles of data distribution is normally distributed it will correlate linearly with a theoretical z value of the quantiles. If the plot of quantiles of the data distribution is not normally distributed, the data points will deviate from the straight line.

The plot can be made on a normal probability paper. But at present computer programs such as SPSS will give an output of the distribution of the sample along with the linear relationship of the normal plot for comparison.

The quantile – quantile (q-q) plot is a graphical technique for determining if the two data sets come from populations with a common distribution.

1.13.14. Gaussian or Normal Distribution: Normal distribution is a probability distribution which describes data which have a symmetric distribution and unimodal. The properties of normal curve is unimodal, perfectly bell shaped, symmetrical curve. The tails of the curve are asymptotic, i.e., get closer and closer to the axis but they never touch it. Mean, median and mode of the distribution coincide. Coefficient of skewness is zero.

1.13.15. Standard Error: Standard erroris defined as the standard deviation of the sampling distribution. The standard error of mean is the standard deviation of the sampling distribution of sample means. The standard error of standard deviation is the standard deviation of the sampling distribution of sample standard deviation.

When population standard deviation (σ) is known, standard error (SE) of mean ($\sigma_{\bar{x}}$), $\sigma_{\bar{x}} = \sigma/\sqrt{n}$ If σ is not known $S\bar{x} = S/\sqrt{n}$; where $S = \sqrt{\frac{\sum(x-\bar{X})^2}{n-1}}$

1.13.16. Student's t - Test: W. S. Gosset described a distribution called t distribution which enables to make reasonably valid inferences about the population using statistic from small samples. It is commonly referred to as the t-distribution and the tests of significance based on it are called the t- tests (Altman, 1991). This test requires the variables is approximately normally distributed and have equal variances.

F test or variance ratio test: F test is any test in which the test statistic has an F distribution under the null hypothesis. The variance ratio is the ratio of the sample variances or the square of the ratio of the sample standard deviations. The t test is based on the assumption that the two population variances are the same, so we test the null hypothesis by F test (levene's test). Levenes test is used before a comparison of means. In ANOVA, equal variance across sample is tested by homogeneity of variance test. These tests provide an F statistic and a significant

value (P value) and if this value is less than the critical value of 0.05 then the variances are not equal.

1.13.17. Mann Whitney U test: is a non parametric alternative to the t test for comparing data from two independent groups. This test requires all the observations to be ranked as if they were from a single sample. The test involves the calculation of statistic U, and is more complicated and calculated as: $U = n_1 n_2 + (n_1 + 1) - T$.

1.13.18. One way ANOVA: One way ANOVA is used for comparison of means of several groups. The principle behind analysis of variance is to partition the variability of a set off data into components due to different sources of variation. The main assumptions underlying the use of ANOVA are as follows:

- Individuals in various groups are selected on the basis of random sampling
- Variables should follow normal distribution.
- Variables of the groups should be homogeneous. This should be tested by using variance ratio test.
- Samples comprising the groups should be independent.

The total variability of the data is measured by the total sum of squares, this total is partitioned into a) the **with in** groups sum of squares and b) the **between groups** sum of squares. Each sum of squares is converted into an estimated variance (**mean square**) by dividing by its degree of freedom.

$$F = \frac{\text{mean sum of squares between groups}}{\text{mean sum of square with in groups}}$$

Kruskal – Wallis test: is a non parametric test, and is used when the assumption of ANOVA are not met. It is a mathematical extension of Mann Whitney U test. The kruskal - Wallis test statistic is approximately a chi-square distribution, with k-1 degree of freedom. The calculation of the test statistic is simple. The complete set of N observations are ranked from 1 – N regardless of which group they are in, and for each group the sum of the rank is calculated. If the sum of the ranks of n_i observations in the i^{th} group is $R_{i,}$. We calculate the statistic H defined by

$$H = \frac{12}{N(N+1)} \Sigma \frac{R_i^2}{n_i} - 3(N+1)$$

1.13.19. Multiple comparisons: multiple comparison tests are used to interpret significant variation among the means of three or more groups. If we perform k paired comparisons, then we should multiply the P value obtained from each test by k; that is we calculate P'= kP with the restriction that P' cannot exceed 1. This simple adjustment is known as **Bonferroni method**. Bonferroni correction is used to counteract the problem of multiple comparisons. For small numbers of comparison its use is reasonable, but for large number it is highly conservative.

Tukey's HSD test is a single step multiple comparison procedure. It is used in ANOVA (post-hoc analysis) to find means that are significantly different from each other. The Tukey method is used when there are unequal sample sizes. Tukey's test is based on a formula very similar to that of a t test.

1.13.20. Correlation: The relationship between two or more variables is called "correlation", and the variables are said to be correlated. The relationship between two variables is also known as co variation.

Correlation between variables may be either simple or multiple. A simple correlation deals with two variables whereas multiple correlation deals with more than two variables. Correlation between two variables may be positive correlation or negative correlation.

A correlation between two variables in which, with an increase in the value of one variable the value of other variable also increases, and with a decrease in the value of one variable the value of the other variable also decreases, is said to be a positive correlation. In positive correlation the values of both variables move in the same direction.

A correlation between two variables in which when there is an increase in the values of one variables, the values of the other variable decreases, and when there is a decrease in the values of one variable the other variable increases , is said to be a negative correlation. The values of two variables are move in opposite direction in negative correlation.

The two method used to study the presence or absence and extend of correlation between variables are scatter diagram and Karl Pearson's coefficient of correlation (Altman, 1991). Karl Pearson's Coefficient of Correlation is a measure of the linear correlation between two variables X and Y, giving a value between +1 and -1 inclusive, where 1 is total positive correlation, 0 is no correlation and -1 is total negative correlation. It is widely used as a measure of the degree of linear dependence between two variables (Altman, 1991).

Speaman's rank correation: The spearman's rank correlation is a non parametric version of the Pearson correlation. Spearman's correlation coefficient measures the strength and direction of association between two rank variables. Spearman's rank correlation coefficient is exactly the same as the Pearson correlation coefficient calculated on the ranks of the observations. If the data are not near to normal distribution for either variable, it is preferable to use rank correlation.

X-Y Scatterdiagram: It is an easy and simple method for studying correlation between two variables. If X and Y are pairs of variables, the values of the variable X are marked in the X – axis and the values of variable Y are marked in the Y axis. A point is plotted against each value of X and the corresponding Y value. A swarm of dots is obtained, and this is called the scatter diagram. From this scatter diagram we can understand about the correlation between the variables whether it is positive correlation or negative correlation.

If the dots are scattered around a straight line running from left to right in an upward direction, then the correlation between the two variables is said to be positive. A scatter diagram in which the plotted dots form a swarm around a straight line that runs from left to right in the downward direction indicates negative correlation between the variables.

Chapter 2

GENERAL MATERIALS AND METHODOLOGY

2.1. Study Settings and Design

Healthy cross section of participants (n = 80 to 136) between 18 and 25 years, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala Institute of Medical Sciences and Amala Cancer Research Centre, from the rural population of Central Kerala.

The Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011) approved the study. Informed written consent was obtained from each participant who took part in the study.

2.2. Selection of participants, Inclusion and Exclusion criteria

Volunteers underwent a clinical evaluation for inclusion of individuals without any disease conditions or injury; were on regular diet, exercise, rest and sleep; had no drugs for one week and all female participants were in the pre gestational period. Clinical Biochemistry laboratory evaluation was done for further exclusion of unhealthy individuals at the subclinical level and for evaluating the characteristics of diabetes-related variables.

Exclusion criteria for these clinical parameters were: BMI >30 kg/m^2, family history suggestive of obesity and type 1 diabetes, serum triglyceride >250 mg/dl (2.825 mmol/l), waist circumference ≥100 cm, fasting glucose ≥126 mg/dl (7 mmol/L), 2 hour glucose challenged or postprandial glucose >180 mg/dl (10 mmol/L), BP ≥140/90, serum alanine aminotransferase above 125 U/L, hsCRP >5 mg/l, serum creatinine >1.3 mg/dl (114.9 μmol/L) in males and >1.2 mg/dl (106.1 μmol/L) in females. These abnormal cut off levels were designed for this study and permitted inclusion of individuals with consequences of increased insulin resistance and family history of type 2 diabetes mellitus but excluded the secondary clinical influences.

2.3. Sample collection

Blood samples (10 ml) were drawn without anticoagulants, after a 10 to 12 hour overnight fasting, between 7.30 and 8.30 in the morning. Samples were centrifuged immediately in plastic tubes to sediment cells before clotting. Plasma was transferred to glass tubes for clotting and clot was separated by a second centrifugation. If clotting was observed after the first centrifugation, then plasma was allowed to clot in the same tube and then centrifuged. This procedure reduced hemolysis. Cell lysis caused insulin degradation. All assays were done immediately after preparation of serum.

2.4.Sample Size

In this study, we expected to decrease the mean and SD, and thus the reference interval for each analyte. So literature reference could not be used to determine the sample size. A minimum samlple size of 30 represents the population. Sample size of 40 is convenient for plotting the normal distribution curve. The final sample sizes were determined after examining the preliminary results. Sample sizes were calculated from mean and standard deviation.

2.5. Measurement of Body mass index

Body mass index (BMI) is accepted as a better estimate of body fatness and health risk than body weight.

$$\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height (metre)}^2}$$

2.6. Measurement of Waist circumference

WC measurements should be made around a patient's bare midriff, after the patient exhales while standing without footwear and with both feet touching and arms hanging freely. The measuring tape should be made of a material that is not easily stretched, such as fiberglass. In a research background, WC measurements are typically taken 3 times and recorded to the nearest 0.1 cm.

2.7. Autoanalysers for Chemistry and Immunochemistry Parameters

Biochemical analysis was done by immunochemistry autoanalyser Vitros ECi (Ortho Clinical Diagnostics, USA), Access 2 (Beckman Coulter, USA) and Liaison, Diasorin, Italy. Chemistry autoanalyser was Vitros 5, 1 FS (OCD, USA) with both dry chemistry and wet chemistry sections.

2.8. Detailed Description of an Immunochemistry Assay, eg. Insulin Assay

Insulin assays were done with Access 2 machine and reagents, (Beckman Coulter, USA) using immunometric assay with magnetic bead coated antiinsulin antibody (Ultrasensitive insulin, 2005). Limit of detection of insulin was taken (Linnet & Boyd, 2012) as the lowest insulin concentration distinguishable from zero (calibrator as 0 pmol/L insulin) with 95% confidence, and was 0.21 pmol/L (Ultrasensitive insulin, 2005). This was far below, the lower limit of range of fasting insulin in this study. It was also below the lowest calibrator after zero, of the linear six point insulin calibrator value (an example from a lot is 5.88 pmol/L). An example of actual linear six point calibration values for insulin in pmol/l for a particular lot of calibrators were 0, 5.88, 58.2, 291.6, 888, 1818. Insulin in μU/ml x 6 converted it to pmol/L (Heinemann, 2010). The two level internal quality control reagents and the external quality assurance programs were from Biorad Laboratories, Irvine, CA, USA. Claims of the manufacturers of instruments and reagents were validated by internal quality control samples assayed in duplicates for a minimum of ten days or often more. Cross reactivity of assayed serum insulin with proinsulin and C peptide were insignificant (Ultrasensitive insulin, 2005). Even weak hemolysis in sample showed marked decrease in insulin due to degradation (Ultrasensitive insulin, 2005, Heinemann, 2010) and was the most important interference in the assay. Reference interval of fasting insulin (manufacturer's) was 11.4 to 138 pmol/L (Ultrasensitive insulin, 2005).

Sandwich or immunometric immune assay was done using mouse monoclonal anti insulin antiboldy coated on paramagnetic particles and mouse monoclonal anti insulin antibody conjugated with alkaline phosphatase. Detection was by chemiluminescence using chemiluminence reagents and luminometer (Allauzen et.al, 1994; Allauzen et.al, 1995).

1. Sample was added to reaction vessel.
2. Mouse monoclonal anti-insulin alkaline phosphatase conjugate was added along with the sample.
3. Paramagnetic particles coated with mouse monoclonal anti-insulin antibody were also added to the reaction vessel.
4. After incubation in the reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away.
5. Then, the chemiluminiscent substrate Lumi-Phos*530 was added to the vessel.
6. Light generated by the reaction was measured with a luminometer.
7. The light production is directly proportional to the concentration of insulin in the sample.

2.9. Assay of C peptide

The autoanalyser used for immunochemistry estimation of serum C peptide was Liaison, Diasorin, Italy, using their reagents. Sandwich chemiluminescence immnoassay was done using mouse monoclonal anti C peptide antibody coated on the magnetic particles (Solid Phase) and another monoclonal antibody is linked to an isoluminol-antibody conjugate.

During the incubation, C peptide present in samples binds to the solid phase monoclonal antibody, and subsequently the antibody conjugate reacts with C peptide already bound to the solid phase. After incubation the unbound materials is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescence reaction is thus induced. The light signal, and hence the amount of isoluminol-antibody conjugate, is measured by a photomultiplier as relative light units (RLU) and is indicative of C peptide concentration present in samples.

2.10. Detailed Description of a Chemistry Assay: eg. Glucose Assay

Assay was done on a Chemistry autoanalyser, Vitros 5, 1 FS (OCD, USA), with a wet chemistry and dry chemistry sections. VITROS glucose slide is a multilayered, analytical element coated on a polyester support.A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the

underlying layers. The oxidation of sample glucose is catalyzed by glucose oxidase to form hydrogen peroxide and gluconate. This reaction is followed by an oxidative coupling catalyzed by peroxidase in the presence of dye precursors to produce a dye. The intensity of the dye is measured by reflected light at 540 nm (Tietz, 1994; Trinder, 1969).

$$\beta\text{-D-glucose} + O_2 + H_2O \longrightarrow \text{D-gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{1,7-dihydroxynaphthalene} \xrightarrow{\text{Peroxidase}} \text{Red dye}$$

Assay type was colorimetric and detection by reflectance spectrophotometry; Approximate Incubation Time: 5 minutes; Temperature of incubation: 37^{o}C; Wavelength: 540 nm; Sample drop volume: 10 µL.

Assay calibration was performed using calibrators of the same lot number as the assay reagent pack. Two point calibrations were done. Level 1: 105.5 mIU/L; Level 2: 453.4 mIU/L. The Master Plot of the calibration plot was automatically aligned with the calibration values obtained. Reportable range: 20 - 625 mg/dl.

Slide Components are mainly made of 5 parts in a slide. They are:

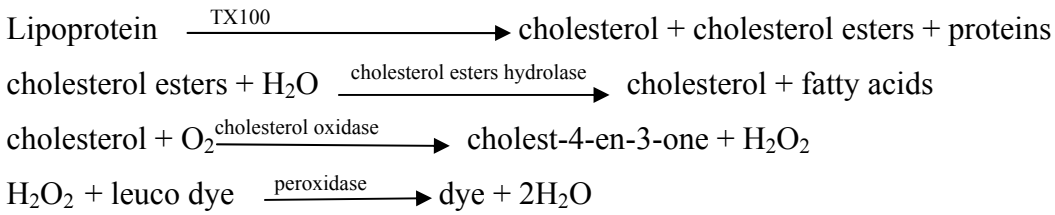
1. Upper slide mount
2. Spreading layer (TiO_2)
3. Reagent layer
    - Glucose oxidase
    - Peroxidase
    - Dye precursors
    - Buffer. pH 5.0
4. Support layer
5. Lower slide mount

2.11. Assay of Cholesterol

Cholesterol assay was performed in chemistry autoanalyser Vitros 5, 1 FS (OCD, USA) using the VITROS dry chemistry slides, which is a multilayered, analytical element coated on polyester support. This is an enzymatic method.A

drop of sample (10µL) is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The Triton X-100 (TX100) surfactant in the spreading layer helps in the dissociation of cholesterol and cholesterol esters from lipoprotein complexes present in the sample. Hydrolysis of the cholesterol esters to cholesterol is catalyzed by cholesterol ester hydrolase. Free cholesterol is then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye. The density of the dye formed is proportional to the cholesterol concentration present in the sample and is measured by reflectance spectrophotometry (Allain et al, 1974). This colorimetric method requires 37^{o}C temperature and an incubation time of 5 minutes. The wave length used is 540nm.

Reaction sequence



Reagents

Triton X-100 - 0.81mg

Cholesterol oxidase - 0.4U (Nocardia or Cellulomonas, E.C.1.1.3.6)

Cholesterol ester hydrolase - 2.0U (Pseudomonas, E.C.3.1.1.13)

Peroxidase - 5.3 U (horseradish root, E.C.1.11.1.7)

2-(3, 5-dimethoxy-4-hydroxyphenyl)-4, 5-bis-

(4-dimethylaminophenyl) imidazole (leuco dye) - 0.17mg.

Pigments, binder, buffer, surfactants, stabilizers and cross-linking agent

2.12. Assay of Direct HDL Cholesterol

HDL (High Density Lipoprotein) cholesterol estimation was performed in chemistry autoanalyser Vitros 5, 1 FS (OCD, USA) using the VITROS dHDL slides. The VITROS dHDL slide is a multilayered, analytical element coated on polyester support. The method is based on a non-HDL precipitation method followed by an enzymatic method. A drop of sample (10µL) is deposited on the

slide and is evenly distributed by the spreading layer to the underlying layers. HDL is separated by the precipitation of non-HDL using phosphotungstic acid (PTA) and magnesium chloride ($MgCl_2$) in the spreading layer. The Emulgen B-66 surfactant in the spreading layer helps in the dissociation of cholesterol and cholesterol esters from HDL lipoprotein complexes present in the sample. Hydrolysis of the HDL- derived cholesterol esters to cholesterol is catalyzed by cholesterol ester hydrolase. Free cholesterol is then oxidized in the presence of cholesterol oxidase to form cholestenone and hydrogen peroxide. Finally, hydrogen peroxide oxidizes a leuco dye in the presence of peroxidase to generate a colored dye. The density of the dye formed is proportional to the HDL cholesterol concentration present in the sample and is measured by reflectance spectrophotometry. This colorimetric method requires 37^{o}C temperature and an incubation time of 5 minutes. The wave length used is 670nm (Warnick et al.1983).

Reaction sequence

HDL + non-HDL $\xrightarrow{\text{PTA/ MgCl2}}$ high density lipoproteins + non-HDL

high density lipoprotein $\xrightarrow{\text{Emulgen B-66}}$ cholesterol + HDL cholesterol esters + proteins

cholesterol esters + H_2O $\xrightarrow{\text{cholesterol esters hydrolase}}$ cholesterol + fatty acids

cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ cholest-4-en-3-one + H_2O_2

Reagents

Emulgen B-66 - 0.7mg

Phosphotungstic acid - 0.3mg

Magnesium chloride - 0.2mg

Cholesterol oxidase - 0.8U (Cellulomonas, E.C.1.1.3.6)

Cholesterol ester hydrolase - 1.2U (Candida rugosa, E.C.3.1.1.3)

Peroxidase - 2.2 U (horseradish root, E.C.1.11.1.7)

2-(3, 5-dimethoxy-4-hydroxyphenyl)-4, 5-bis-(4-dimethylaminophenyl)imidazole(leucodye) - 0.02mg.

Pigments, binder, buffer, surfactants, stabilizers, scavenger and cross-linking agents.

2.13 Assay of Triglyceride

Triglyceride estimation was performed in chemistry autoanalyser Vitros 5, 1 FS (OCD, USA) using the VITROS slides. The VITROS slide is a multilayered, analytical element coated on polyester support. This is an enzymatic method. A drop of sample (10μL) is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The Triton X-100 (TX100) surfactant in the spreading layer helps in the dissociation of triglyceride from lipoprotein complexes present in the sample. The triglyceride molecules are then hydrolysed by lipase to yield glycerol and fatty acids. Glycerol diffuses to the reagent layer, where it is phosphorylated by glycerol kinase in the presence of ATP. In the presence of L-α- glycerol-phosphate oxidase, L-α- glycerophosphate is then oxidized to dihydroxyacetone phosphate and hydrogen peroxide. The final reaction involves the oxidation of a leuco dye by hydrogen peroxide, catalyzed by peroxidase, to produce a dye. The density of the dye formed is proportional to the triglyceride concentration present in the sample and is measured by reflectance spectrophotometry (Spayd et al, 1978). This colorimetric method requires 37^{o}C temperature and an incubation time of 5 minutes. The wave length used is 540 nm.

Reaction sequence

Lipoprotein $\xrightarrow{\text{surfactant}}$ triglycerides + proteins

Triglycerides + H_2O $\xrightarrow{\text{lipase}}$ glycerol + fatty acids

Glycerol + ATP $\xrightarrow[MgCl2]{\text{glycerol kinase}}$ L-α- glycerophosphate + ADP

L-α- glycerophosphate + O_2 $\xrightarrow{\text{L-α- glycerol-phosphate oxidase}}$ dihydroxyacetone phosphate + H_2O_2

H_2O_2 + leuco dye $\xrightarrow{\text{peroxidase}}$ dye + $2H_2O$

Reagents

Lipase - 0.15U (Candida rugosa, E.C.3.1.1.3)

Peroxidase - 0.52 U (horseradish root, E.C.1.11.1.7)

Glycerol kinase - 0.35U (Cellulomonas sp., E.C. 2.7.1.30)

L-α- glycerol-phosphate oxidase - 0.19U (Pediococcus sp., E.C.1.1.3.-)

Triton X-100 - 0.62mg

2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis-
(4-dimethylaminophenyl) imidazole (leuco dye) - 0.04mg.

Adenosine triphosphate - 0.14mg

Pigments, binder, buffer, surfactants, stabilizers, scavenger, enzyme cofactor, dye solubilizer and cross-linking agents.

2.14. Estimation of LDL Cholesterol

Both indirect and direct methods are used to measure LDL (Low-Density Lipoprotein) cholesterol. Here we used the indirect method for the estimation of LDL cholesterol. Indirect method assumes that total cholesterol is primarily composed of cholesterol on VLDL, LDL and HDL. LDL cholesterol is then measured indirectly by using Friedewald equation. Here total cholesterol, triglyceride and HDL cholesterol are measured and LDL cholesterol is calculated from the primary measurement by using the empirical Friedewald equation:

LDL cholesterol = [Total cholesterol] – [HDL cholesterol] – [Triglyceride] / 5

where all concentrations are given in milligrams per deciliter.

Triglyceride/2.22 is used when LDL cholesterol is expressed in millimoles per liter. The factor [Triglyceride]/5 is an estimate of VLDL cholesterol concentration and is based on the average ratio of triglyceride to cholesterol in VLDL (Friedewald et al, 1972).

2.15. Assay of Alanine Aminotransferase

ALT estimation was performed in Ortho-Clinical Diagnostics 5.1 Vitros instrument using the VITROS ALT slides. The VITROS ALT slide is a multilayered, analytical element coated on polyester support. This is an enzymatic method. A drop of sample (11 µL) is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. The spreading layer contains the ALT substrates L-alanine and sodium α-ketoglutarate. Alanine aminotransferase catalyzes the transfer of the amino group of L-alanine to α-

ketoglutarate to produce pyruvate and glutamate. Lactate dehydrogenase (LDH) then catalyzes the conversion of pyruvate and NADH to lactate and NAD+. The rate of oxidation of NADH is monitored by reflectance spectrophotometry. The rate of change in reflection density is proportional to enzyme activity. The slide is incubated at 37^{o}C for 5 minutes. The wave length used is 340nm.

Reaction sequence

alanine + α-ketoglutarate $\xrightarrow{\text{ALTpyridoxal-5-phosphate}}$ pyruvate + glutamate

pyruvate + NADH + H^{+} $\xrightarrow{\text{LDH}}$ lactate + NAD+

Reagents

Lactate dehydrogenase (Porcine muscle) – 0.12 U

L-alanine - 0.86 mg

Sodium α-ketoglutarate - 54µg

Nicotinamide adenine dinucleotide, reduced - 35µg

Sodium pyridoxal-5-phosphate - 11µg

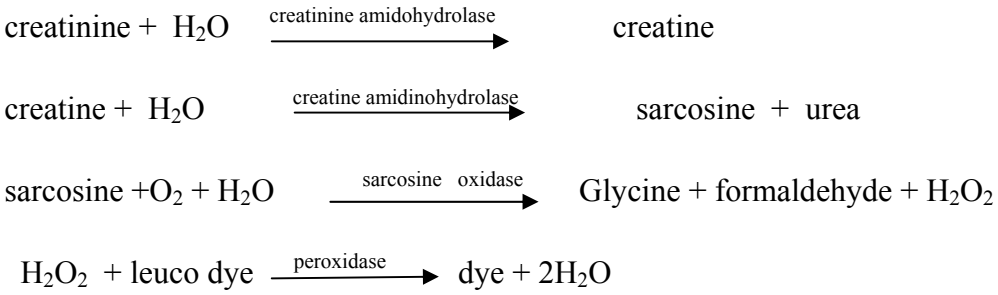
Pigments, binder, buffer, surfactants, stabilizers, and cross-linking agents.

2.16. Assay of Creatinine

Creatinine estimation was performed in Ortho-Clinical Diagnostics 5.1 Vitros instrument using the VITROS CREA slides. The VITROS CREA slide is a multilayered, analytical element coated on polyester support. This is an enzymatic method. A drop of sample (10 µL) is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. Creatinine diffuses to the reagent layer, where it is hydrolyzed to creatine in the rate determining step. The creatine is converted to sarcosine and urea by creatine amidinohydrolase. The sarcosine, in the presence of sarcosine oxidase, is oxidized to glycine, formaldehyde, and hydrogen peroxide. The final reaction involves the peroxidase-catalyzed oxidation of a leuco dye to produce a colored product. Following addition of the sample, the slide is incubated at 37^{o}C for 5 minutes. During the

initial reaction phase, endogenous creatine in the sample is oxidized. The resulting change in reflection density is measured at 2 time points. The difference in reflection density is proportional to the concentration of creatinine present in the sample. The wavelength used is 670 nm.

Reaction sequence



Reagents

Creatine amidohydrolase (Flavobacterium sp.) – 0.20 U

Creatine amidinohydrolase (Flavobacterium sp.) – 4.7 U

sarcosine oxidase (Bacillus sp.) – 0.55 U

Peroxidase (Horseradish root) – 1.6 U

2-(3,5-dimethoxy-4-hydroxyphenyl)-4,5-bis-(4-dimethylaminophenyl)imidazole(leucodye) - 32 μg.

Pigments, binder, buffer, surfactants, stabilizers, scavenger, chelator, dye solubilizer, and cross-linking agents.

2.17. Assay of hsCRP

The quantitative measurement of C-reactive protein (CRP) is performed using the VITROS chemistry products hsCRP reagent in conjunction with the VITROS chemistry products calibrator Kit 17 and VITROS chemistry products FS calibrator 1 on Ortho-Clinical Diagnostics VITROS 5,1 FS chemistry system. hsCRP reagent is a dual chambered package containing ready to use liquid reagents. Samples, calibrators and controls are mixed with Reagent 1 containing a buffer. Addition of anti-CRP antibodies coupled to latex microparticles (Reagent 2) produces a immunochemical reaction yielding CRP antigen/antibody complexes. The turbidity is measured spectrophotometrically at 660 nm. Once a calibration has been performed for each reagent lot, the CRP concentration in each

unknown sample can be determined using the stored calibration curve and the measured absorbance obtained in the assay of the sample. This is a two-point test type and is incubated at 37^{o}C for 8 minutes. Sample volume used is 16 µL.

Step 1: Dilution of sample

C-reactive protein in sample $\xrightarrow{R1}$ C-reactive protein in solution

Step 2: Formation of Antigen-antibody complex

C-reactive protein in solution $\xrightarrow{R2}$ C-reactive protein and antibody particle complex.

Reagents, Reactive ingredients

Reagent 1(R1): none

Reagent 2 (R2): latex particles coated with anti- CRP mouse monoclonal antibodies 0.1% [w/w]

Other ingredients

Reagent 1(R1): buffer, BSA (bovine serum albumin), polmer and preservative

Reagent 2 (R2): buffer and preservative

2.18. Diagnostic criteria

Diagnostic criteria for diabetes laid by the WHO were with fasting plasma glucose ≥126 mg/dL (7 mmol/L) or 2 hour postprandial or 2 hour post glucose load (75 g in 300 ml water for adults) glucose value ≥200 mg/dL (11.1mmol/L) (22). Diabetes mellitus type 2 was differentiated from type 1, by the former having a minimum history of 6 months of glycemic control by drugs, diet and exercise before insulin injection.

Participants in the sample population were partitioned into those without and with family history of type 2 diabetes mellitus in parents.

Diagnostic criteria for diabetes laid by the WHO were with fasting plasma glucose ≥7mmol/l or 2 hour post-glucose load (75g in 300ml water for adults) value ≥11.1mmol/l(Alberti & Zimmet,1998).

DM2 was differentiated from type 1, by the former having history of a minimum of 6 months duration of glycemic control by drugs, diet and exercise before insulin injection.

Body mass index (BMI) and waist circumference were defined by the revised criteria for Asian Indians (WPRO.WHO, 2000; Misra et al.2009) as underweight of <18.5kg/m^2, normal range of 18.5 - 22.9kg/m^2, overweight of 23 - 24.9kg/m^2, obese I of 25 - 29.9kg/m^2, obese II ≥30kg/m^2 for both males and females. A person was considered to have abdominal obesity if the waist circumference ≥ 90 cm for males and ≥80cm for females.

Daily continuous internal quality control data were analysed according to Westgard rules for acceptance or rejection of analyte data (Klee & Westgard, 2012). If there is a rejection, appropriate measures were taken to set right errors in machine functioning, reagents or calibration levels.

2.19. Statistical Analysis

Statistical analysis and calculations were done with SPSS, Minitab and Medcalc software or manually. Normality of distribution was estimated by graphical and numerical tests. Graphical tests used are histogram, Box-Whisker Plot and normal quantile-quantile plot. Normality of distribution was numerically estimated by Kolmogorov-Smirnova, Shapiro-Wilk test, Anderson - Darling normality test and D'Agostino – Pearson tests. Equality or homogeneity of variances of the groups compared was done by Levene's test. Log_{10} transformations converted most of the positively skewed groups to Gaussian distribution and were of equal variances in the groups compared. When variables had Gaussian distribution (before or after transformation) and when there was equality of variance in the groups compared, parametric methods of analysis were used. Otherwise, non parametric methods were used. The significant differences of variables of different groups were compared by 95% confidence interval of mean, two-tailed Student's t test, Mann Whitney U test and one way analysis of variance (ANOVA) with post hoc pair comparison using Tukey HSD for unequal sample number and Bonferroni's correction for multiple comparisons (Klee & Westgard, 2012; Riffenburgh, 2012; Altman, 1991). The relationship between twovariables is done by Pearson correlation, only if one of the variables had Gaussian distribution otherwise non

parametric Spearman's Rank correlation was done. Correlation between the two variables were plotted graphically by X-Y scatter plot. Odds ratio was also done to calculate the risk by using contingency truth table.

2.20. Conversion factors for inter conversion between conventional unit and SI Unit

Analyte	Conventional Unit	Conversion Factor	SI Unit
C-peptide	ng/mL	0.33	nmol/L
Insulin	µIU/mL	6.0	pmol/L
Glucose	mg/dL	0.056	mmol/L
Triglycerides	mg/dL	0.0113	mmol/L
Cholesterol	mg/dL	0.0259	mmol/L
HDL cholesterol	mg/dL	0.0259	mmol/L
Alanine aminotransferase (ALT)	units/L	1.0	U/L
Creatinine	mg/dL	88.4	µmol/L

Chapter 3

Partitioning of fasting and post glucose load oral glucose tolerance test insulin according to family history of type 2 diabetes mellitus in young healthy adults

3.1.Abstract

The level of insulin circulating in plasma is influenced by insulin resistance, hepatic insulin uptake and insulin secretion. Family history of type 2 diabetes mellitus has been reported to be related to hyperinsulinemia. In this study, the relationship of fasting insulin with post glucose load oral glucose tolerance (OGTT) insulin levels followed by partitioning of fasting and OGTT insulin according to family history of type 2 diabetes mellitus. Clinical and biochemical evaluation of volunteers were done for inclusion as participants (n = 90) or their exclusion. Cut-off levels of quantitative biochemical variables were fixed such that they included the effects of insulin resistance and beta cell dysfunction. Distribution was analysed by Shapiro-Wilk test; Equality of variances by Levene's test; Log_{10} transformations for conversion of groups to Gaussian distribution and for equality of variances in the groups compared. When the groups compared had Gaussian distribution and there was equality of variance, parametric methods were used. Otherwise, non parametric methods were used.Fasting insulin was correlating significantly with 30, 60, 90 and 120 minute OGTT insulin showing that hyperinsulinemia in the fasting state was related to hyperinsulinemia in the post glucose load states. When fasting and post glucose load OGTT insulin were partitioned into those without and with family history of type 2 diabetes, maximum difference was seen in fasting insulin ($P < 0.001$), followed by 120 ($P = 0.001$), 60 ($P = 0.002$) and 90 ($P = 0.017$) minute OGTT insulin. The 30 minute insulin could not be partitioned ($P = 0.574$). Partitioning fasting insulin according to family history of type 2 diabetes mellitus is a prerequisite for establishment of baseline reference intervals of partitioned groups, which in turn are required for its clinical interpretation.

3.2.Introduction

Type 2 diabetes mellitus is the consequence of multiple genetic and environmental influences in the individual. Though genetic causes are not clear, multiple genes are involved in type 2 diabetes (Herder & Roden, 2011; Gaulton, 2015;Ayub et al, 2014). These genes contributed to increased prevalence of type 2

diabetes mellitus in families. Family history of type 2 diabetes in parents resulted in early fasting hyperinsulinemia in non diabetic offspring (Haffner et al, 1988). The genetic factors also contributed to heritability of insulin secretory deficiency (Elbein et al,1999; Stumvoll et al, 2002) and metabolic changes (Tripathy et al, 2003), resulting in higher prevalence and aggregation of diabetes in families (Valdez et al, 2007), especially among twins (Poulsen et al, 1999). Non genetic inheritance factors may be epigenetic or non epigenetic mechanisms such as familial behavior and culture. The reported increased maternal transmission of diabetes may result from effects of gestational diabetes on fetus or from maternally inherited mitochondrial genes (Dabelea et al, 2000;Fetita et al, 2006).

The multiple genetic influences contributed by the family history of type 2 diabetes mellitus causes stratification of insulin levels, that is, there is hyperinsulinemia with family history. Partitioning a sample population with reference to a biochemical marker is required for clinical analysis and diagnosis, and to make inferences of clinical or subclinical disease states. Fasting insulin is one such intermediate marker. Partitioning the fasting and post glucose load oral glucose tolerance test (OGTT) insulin is required for analysis of their relationships with other biochemical and clinical parameters (Khan, 2003; Gerich, 2003; Weyer et al, 1999), for establishment of the baseline status and the baseline reference intervals of insulin (CLSI, 2008), and to understand the heterogeneity and stratification of the intermediate marker with reference to multiple influences (Horowitz, 2012; Lahti, 2004;Shirts et al, 2011).

The study problem and hypothesis of this section is as follows. Here we explore the relationships of fasting insulin with post glucose load insulin, as both are influenced by family history of type 2 diabetes mellitus. If theydo correlate, then it is likely that both fasting and OGTT insulin may be partitioned according to family history of type 2 diabetes mellitus.

3.3.Aim of study in this section

The relationship of fasting insulin with post glucose load OGTT insulin was analysed in young adults. This was followed by partitioning fasting and post glucose load OGTT serum insulin according to family history of type 2 diabetes.

3.4.Materials and Methods

3.4.1. Study Settings and Design

Healthy cross section of participants (n = 90) between 18 and 25 years, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala from rural population of Central Kerala.

The Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011) approved the study. Informed written consent was obtained from each participant aged between 18 and 25 years.

3.4.2. Selection of participants, Inclusion and Exclusion criteria

Please refer 2.2.Selection of participants, Inclusion and Exclusion criteriain Chapter 2.

3.4.3.Sample collection

Please refer2.3.for sample collection in Chapter 2.

3.4.4.Analytical control and insulin measurements

Please refer 2.8. fordetailed description of an Immunochemistry Assay, eg. Insulin Assay in Chapter 2.

3.4.5. Diagnostic criteria

Please refer 2.18. Diagnostic criteriainChapter 2

3.4.6. Statistical Analysis

Please refer 2.19. Statistical Analysis inChapter 2

3.5. Results

Characteristics of some insulin and diabetes-related quantitative variables in the total sample (n = 90) are given and were evaluated(Table 3.1). The mean values of all quantitative variables were within the healthy reference range. Ranges of all quantitative variables were also within the cut off levels permitted by

the inclusion/exclusion criteria. Most of the data were positively skewed. Peak level of insulin was seen at 30 minute, followed by 60 minute. Insulin level decreased at 90 minute OGTT and even at 120 minute OGTT insulin level was far above fasting levels. Fasting and 120 minute OGTT glucose showed that none of the participants had diabetes.

None of the insulin data before $\log_{10}$ transformation was normally distributed as seen by K-S and Shapiro-Wilk statistic (Table 3.2). The P value ranged from 0.001 to <0.001 showing that the data was significantly different from Gaussian distribution. After $\log_{10}$ transformation, all the insulin data became normally distributed by Shapiro Wilk method and the P value ranged from 0.840 to 0.236.

As the insulin data did not have Gaussian distribution, fasting insulin was analysed by histogram, Q-Q plot and by Box-Whisker plot (Fig. 3.1). The data was found to be positively skewed (Fig. 3.1A), away from Gaussian distribution (Fig. 3.1B) and had seven positive outliers (Fig. 3.1C). Of the seven positive outliers, five were near and two were far outliers (Fig. 1C).

After $\log_{10}$ transformation, fasting insulin was analysed by histogram, Q-Q plot and Box Whisker plot (Fig. 3.2). Then fasting insulin data was found to be normally distributed by histogram (Fig. 3.2A), Q-Q plot (Fig. 3.2B) and by Box-Whisker plot (Fig. 3.2C). The Box Whisker plot showed two near positive outliers and two near negative outliers only.

Calculation of Pearson's correlation coefficient (Table 3.3) and visual examination of X-Y scatter diagram (Fig. 3.3A) showed significant correlation ($r = 0.265$; $P = 0.012$) and direct relationship of fasting insulin and 30 minute insulin. Similarly, there were significant correlations of fasting insulin with 60 minute ($r = 0.335$; $P = 0.001$), 90 minute ($r = 0.393$; $P = <0.001$) and 120 minute ($r = 0.277$; $P = 0.008$) OGTT serum insulin.

Insulin in groups without and with family history of type 2 diabetes in parents was compared (Table 3.4). Fasting insulin in group with family history of diabetes were positively skewed. Log_{10} transformation converted the distribution of most of the samples to Gaussian and had equality of variance. Percent coefficient of

variation (%CV) was found to be lowest in fasting insulin when compared with that of 30, 60, 90 and 120 minute insulin in both groups with and without family history of diabetes (Table 3.4).

Mean fasting insulin was markedly increased in the group with family history of diabetes (P <0.001) but there was no difference in 30 minute insulin in the groups with and without family history of diabetes mellitus (P = 0.574). There was a good increase at 60 (P = 0.002), 90 (P = 0.017) and 120 minute (P = 0.001) OGTT insulin with family history of type 2 diabetes mellitus, when compared with the corresponding group without family history. These results indicated that fasting insulin showed much better difference between those with and without family history of diabetes mellitus.

Similar differences were also seen in 95% CI of mean of serum insulin (Table. 3.5; Fig. 3.4; Fig. 3.5). The 95%CI with and without family history of diabetes were far separated with fasting insulin, moderately separated with 60, 90 and 120 minute insulin, but was overlapping in 30 minute insulin. These results indicated that there is better partitioning of fasting insulin than post glucose load OGTT insulin with family history of type 2 diabetes mellitus. There was no partitioning of 30 minute insulin.

3.6. Discussion

Circulating plasma concentrations of fasting insulin, fasting glucose and release of hepatic glucose are in a steady state. This steady state is influenced by insulin sensitivity, insulin uptake by tissues and insulin secretion by pancreas (Fetita et al,2006; Khan, 2003; Gerich, 2003; Weyer et al, 1999). All these factors also affected post glucose challenged OGTT insulin levels. These relations between fasting insulin and post glucose challenged insulin are responsible for the correlation between them (Table 3.3 and Fig. 3.3).

Hyperinsulinemia is an early clinically detectable parameter. Insulin levels increase much before the onset of type 2 diabetes(Haffner et al, 1988). Hyperinsulinemia was reported to be related to family history of type 2 diabetes mellitus in parents. Partitioning fasting and OGTT insulin levels according to

family history of type 2 diabetes was taken up for this study (Table 3.4 and 3.5; Fig. 3.4 and 3.5).

The two fundamental defects observed in type 2 diabetes mellitus are insulin resistance and beta cell insulin secretory dysfunction. Insulin resistance, increased insulin secretion and beta cell dysfunction decreased insulin secretion (Khan, 2003; Gerich, 2003). Both these activities influence fasting insulin in oppose directions and confound each other. It may be reasoned that this confounding of hyerinsulinemia by beta cell secretory dysfunction is the fundamental problem in clinical interpretation of fasting insulin levels.

There is a large increase in glucose-induced insulin secretion during OGTT when compared to fasting insulin. The ability of beta cells to increase secretion of insulin depends on the status of insulin resistance and compensation of insulin resistance by beta cells (Khan, 2003;Armitage et al, 2002; Prentki & Nolan, 2006). But insulin secretory dysfunction decreased insulin secretion by beta cells. These activities increasing and decreasing insulin secretion act in opposite directions and cause increase %CV of serum insulin. The %CV was found to be lowest in fasting insulin. Therefore, fasting insulin was found to be more reproducible than glucose challenged insulin of OGTT.

Decrease in hepatic insulin clearance of plasma insulin is considered as a cause of hyperinsulinemia (Kasuga, 2006). Hyperinsulinemia may be considered as a compensation for insulin resistance which also increases insulin secretion. Hyperinsulinemia is strongly associated with metabolic syndrome, pathogenesis of type 2 diabetes and obesity (Khan, 2003; Gerich, 2003; Weyer et al, 1999; Armitage et al, 2002; Kasuga, 2006).

To conclude, family history of type 2 diabetes was found to have an influence on serum insulin. The influence was more markedly detected in fasting insulin. The decrease in coefficient of variation, standard deviation and standard error of the observed values can increase the significance of the difference between two mean values. Young adults between 18 and 25 years were selected for this study to decrease the influence of growth phase upto 18 years of age and environmental build up after 25 years of age.

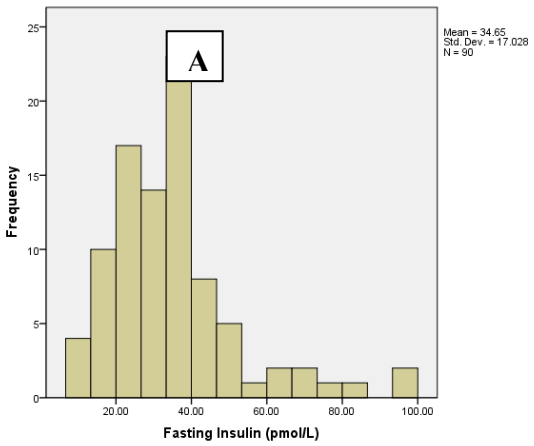
Table 3.1. Characteristics of insulin, OGTT and type 2 diabetes mellitus-related parameters in the total sample of healthy young adults, males and females, with and without family history of type 2 diabetes mellitus in parents (n = 90). OGTT is oral glucose tolerance test. There were four samples missing in the lipid profile data.

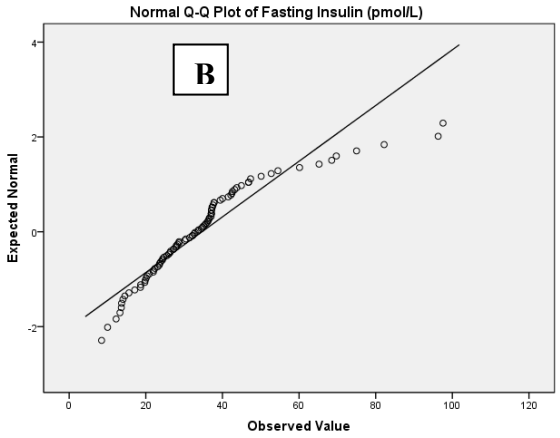
Quantitative variables n = 90 (Standard Units) (Conventional Units)	**Mean±SD in Standard Units (Conventional Units)**	**Range in Standard Units (Conventional Units)**
Age (years)	21.74±2.05	18 – 25
BMI (kg/m^2)	20.71±2.89	15.47 – 27.11
Waist circumference (cm)	75.26±8.51	57.50 – 94.00
Fasting Glucose (mmol/L) (mg/dL)	5.16±0.514 (92.92±9.26)	3.97 - 6.87 (71.50 – 123.70)
2 hour OGTT Glucose, (mmol/L) (mg/dL)	5.67±1.25 (102.25±22.56)	2.49 – 9.76 (44.86 – 175.80)
Fasting Insulin (pmol/L) (µIU/ml)	34.65±17.03 (5.78±2.84)	8.46 – 97.56 (1.41 - 16.26)
30 minute OGTT Insulin (pmol/L) (µIU/ml)	382.40±246.66 (63.73±41.11)	69.48 – 1373.40 (11.58 – 228.90)
60 minute OGTT Insulin (pmol/L) (µIU/ml)	341.66±243.80 (56.94±40.63)	67.32 – 1612.08 (11.22 – 268.68)
90 minute OGTT Insulin (pmol/L) (µIU/ml)	275.37±193.06 (45.90±32.18)	8.52 – 1053.72 (1.42 – 175.62)
120 minute OGTT Insulin (pmol/L) (µIU/ml)	224.24±159.07 (37.37±26.51)	21.06 – 880.50 (3.51 – 146.75)
Total Cholesterol (n = 86) (mmol/L)(mg/dL)	4.58 ±0.727 (176.98±28.05)	2.85 - 6.53 (110.00 – 252.00)
Triglycerides (n = 86) (mmol/L) (mg/dL)	0.880±0.357 (77.88±31.64)	0.42 – 2.51 (37 – 222)
LDL Cholesterol (n = 86) (mmol/L)(mg/dL)	2.84±0.663 (109.67±25.60)	1.40 – 4.84 (54 - 187)
HDL Cholesterol (n = 86) (mmol/L) (mg/dL)	1.34±0.315 (51.67±12.14)	0.73 – 2.20 (28 – 85)

Table 3.2. Distribution of fasting, 30, 60, 90 and 120 minute post glucose load OGTT insulin before and after log_{10} transformation in the total sample (n = 90).

OGTT Insulin (n = 90)	before log_{10} transformation (pmol/L)		after log_{10} transformation	
	Kolmogorov-Smirnova P	Shapiro Wilk test P	Kolmogorov-Smirnova P	Shapiro Wilk test P
Fasting	<0.001	<0.001	0.153	0.236
30 minute	<0.001	<0.001	0.200	0.262
60 minute	<0.001	<0.001	0.132	0.840
90 minute	0.001	<0.001	0.034	0.003
120 minute	<0.001	<0.001	0.200	0.319

Fig. 3.1. Distribution of fasting insulin data before $\log_{10}$ transformation in the total sample. A: Histogram, B: Q-Q plot, C: Box Whisker plot.





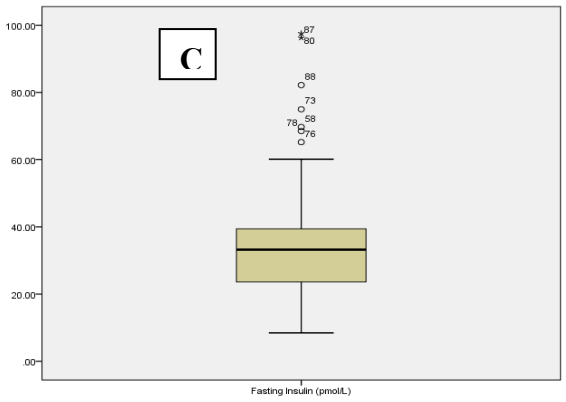
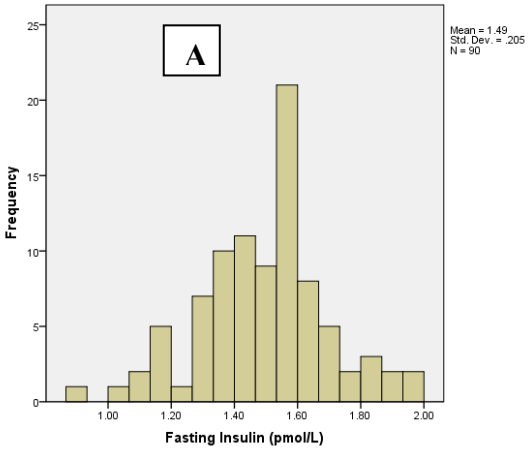
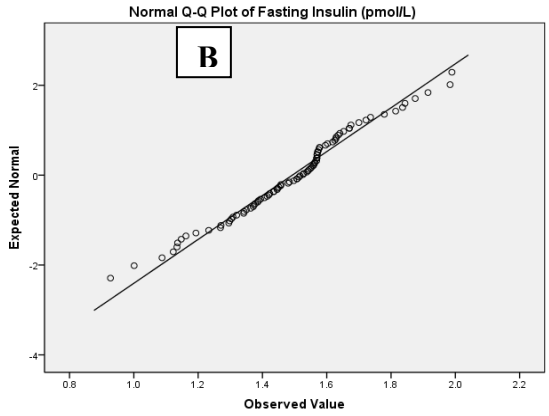


Fig. 3.2. Distribution of Fasting Insulin after $\log_{10}$ transformation of the total sample: A. Histogram, B: Q-Q plot, C: Box Whisker plot.





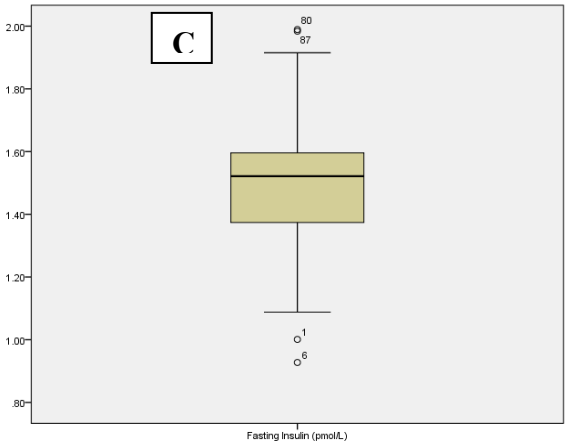


Table 3.3. Pearson's correlation of fasting insulin (Row No. 1) with 30, 60, 90 or 120 minute (Row Nos. 2 – 5) post glucose load OGTT insulin after $\log_{10}$ transformation of insulin data in the total sample. At least one of the two correlating quantitative variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of distribution.

Row No.	Insulin from OGTT (n = 90)	95% Confidence Interval of mean	Shapiro Wilk test (after $\log_{10}$ transformation) P	Correlation coefficient (r)	Significance of correlation P
1.	**Fasting (pmol/L)**	31.08 – 38.22	0.236	--	--
2.	**30 minute (pmol/L)**	330.74 – 434.06	0.262	0.265	0.012
3.	**60 minute (pmol/L)**	290.60 – 392.73	0.840	0.335	0.001
4.	**90 minute (pmol/L)**	234.94 – 315.81	0.003	0.393	<0.001
5.	**120 minute (pmol/L)**	190.92 – 257.56	0.319	0.277	0.008

Fig. 3.3. X-Y scatter diagram of fasting insulin (0 minute) with 30 (A), 60 (B), 90 (C) or 120 (D) minute post glucose load oral glucose tolerance test insulin levels in the total sample.

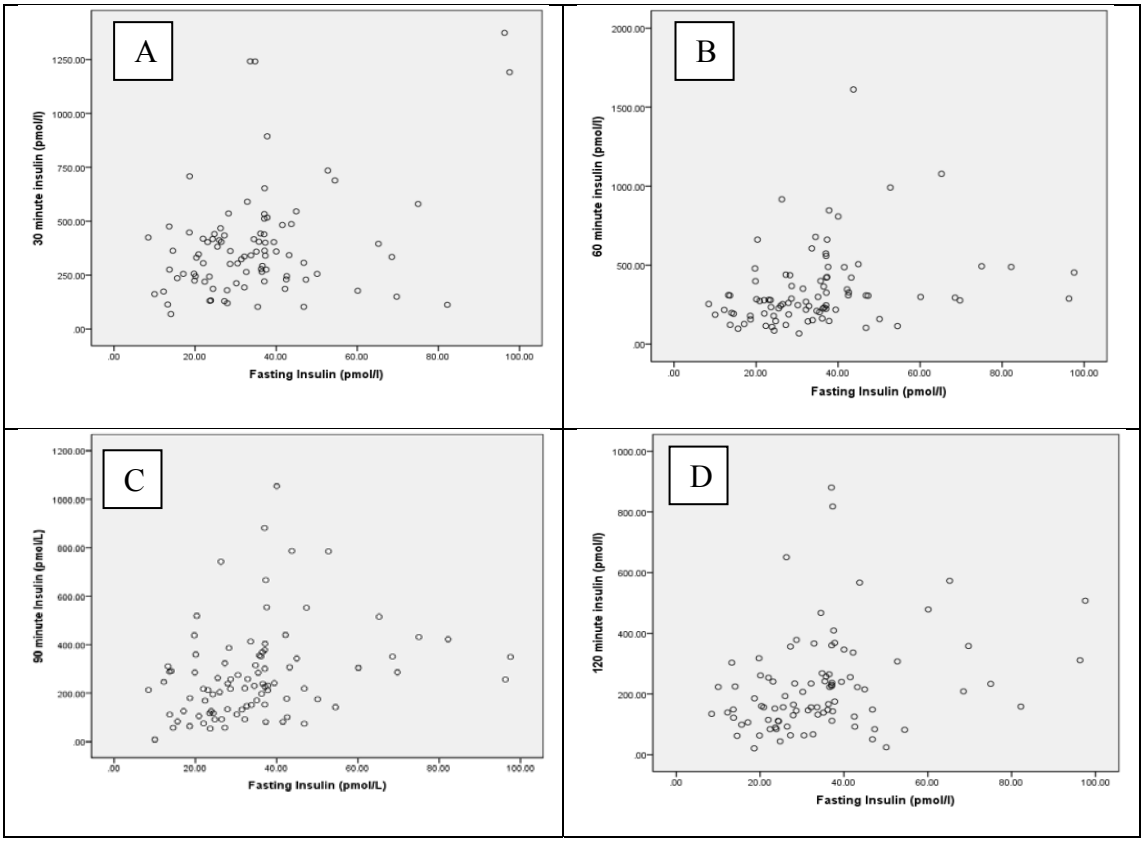


Table 3.4. Comparison of insulin concentrations in groups without and with family history of type 2 diabetes mellitus. As groups were positively skewed, $\log_{10}$ transformation was done. When the insulin data had Gaussian distribution and when there was equality of variance, parametric Student's t test was done. Otherwise, non parametric Mann Whitney U test was done and is given in brackets. %CV is percent coefficient of variation.

Fasting and OGTT Serum Insulin (pmol/ L)	Without family history of type 2 diabetes (n = 57)		With family history of type 2 diabetes (n = 33)		After $\log_{10}$ transformation			
	Mean ±SD (Range)	%CV	Mean ±SD (Range)	%CV	Shapiro-Wilk test for normality P		Levene's test for equality of variances P	Student t test (or Mann-Whitney U test) P
					Without FH	With FH		
Fasting	29.12±10.47 (8.46 - 54.48)	35.95	44.20±21.59 (12.2 - 97.6)	48.84	0.023	0.473	0.491	(<0.001
30 minute	363.98±220.07 (103.2 - 1241.8)	60.46	414.22±287.78 (69.5 - 1373.4)	69.48	0.225	0.940	0.390	0.574
60 minute	281.34±150.15 (67.3 - 661.2)	53.37	445.86±328.91 (121.3 - 1612.1)	73.77	0.680	0.164	0.544	0.002
90 minute	238.60±162.74 (8.52 – 880.86)	68.21	338.89±225.27 (57.90 – 1053.72)	66.47	0.006	0.363	0.358	(0.017)
120 minute	189.88±153.96 (21.06 - 880.5)	81.08	283.59±152.17 (63.96 - 650.7)	53.66	0.180	0.536	0.481	0.001

Table 3.5. Comparison of 95% CI of mean insulin concentrations in groups without and with family history of type 2 diabetes mellitus.

Fasting and OGTT serum Insulin (pmol/L)	95% CI of mean	
	Without family history	With family history
Fasting	26.34 – 31.90	36.55 – 51.86
30 minute	305.59 – 422.37	312.18 – 516.26
60 minute	241.50 – 321.18	329.23 – 562.49
90 minute	195.41 – 281.78	259.02 – 418.77
120 minute	149.03 – 230.73	229.63 – 337.55

Fig. 3.4. Comparison of 95% confidence intervals of mean fasting insulin in groups without and with family history of type 2 diabetes in parents.

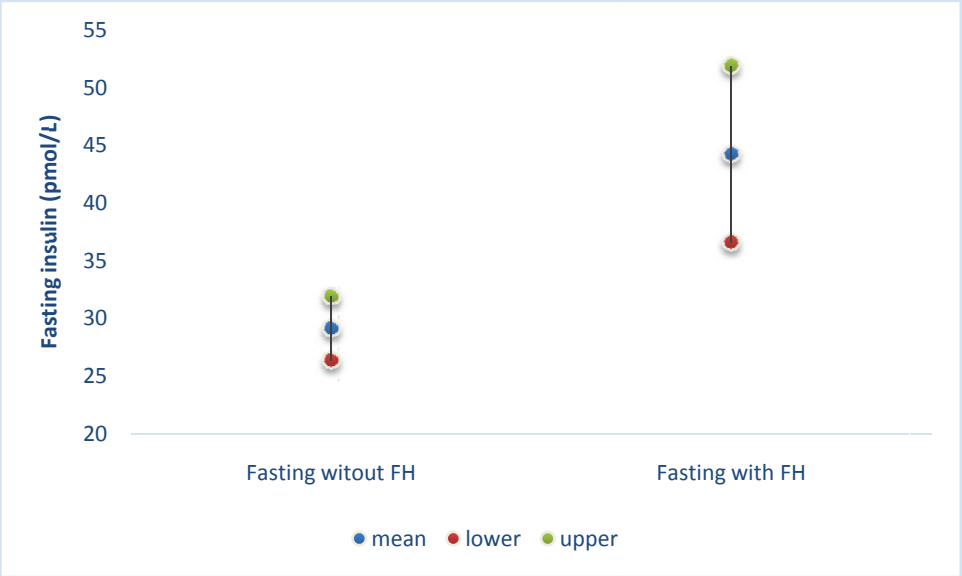
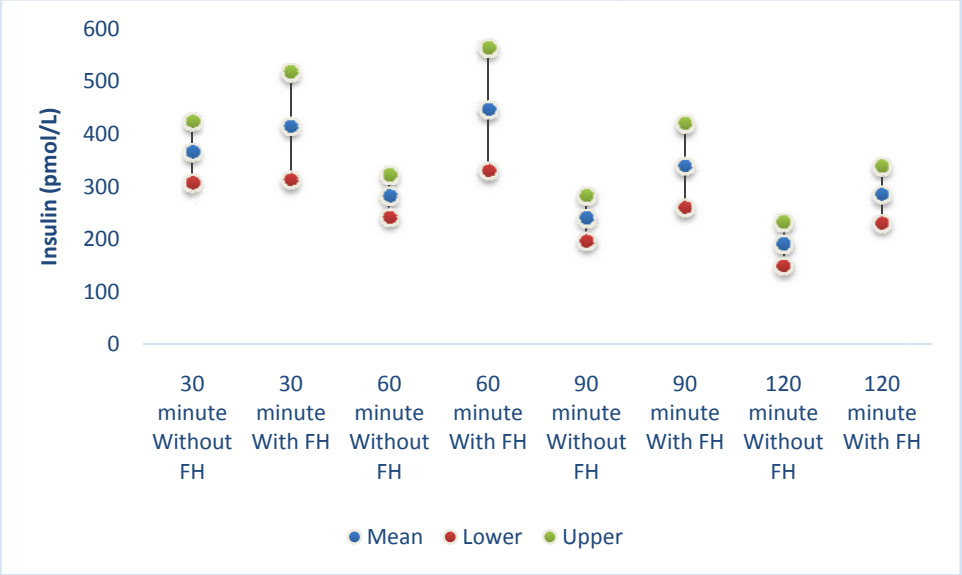


Fig. 3.5. Comparison of 95% confidence intervals of mean of 30, 60, 90 and 120 minute OGTT insulin from sample of participants without and with family history of type 2 diabetes in parents.



Chapter 4

Partitioning fasting and post glucose load OGTT C peptide according to family history of type 2 diabetes mellitus

4.1. Abstract

Insulin resistance and pancreatic beta cell insulin secretory dysfunction are fundamental to the pathogenesis and progress of type 2 diabetes. Insulin resistance causes increase in insulin secretion and beta cell dysfunction decreases it causing difficulties in clinically quantifying the relative levels of these influences. Morning fasting glucose homeostasis is a steady state condition without diurnal influences and C peptide represents insulin secretion. This section of the study analysed the influence of family history of type 2 diabetes on fasting C peptide and oral glucose tolerance test (OGTT) C peptide. Participants of this observational cross sectional study, aged 18 to 25 years from rural Central Kerala, have decreased influence of age, growth phase and environment. Clinical and biochemical evaluation of volunteers were done for inclusion as participants (n = 80) or their exclusion. Cut-off levels of quantitative biochemical variables were fixed such that they included the effects of insulin resistance, but excluded other secondary clinical influences. Distribution was analysed by Shapiro-Wilk test; Equality of variances by Levene's test; Log_{10} transformations were done to convert groups to Gaussian distribution and for equality of variances in the groups compared. When the groups compared had Gaussian distribution and there was equality of variance, parametric methods were used. Otherwise, non parametric methods were used. FastingC peptide correlated well with post glucose load OGTT C peptide or with fasting insulin and triglycerides. Correlations were better with 30 minute OGTT C peptide than with 60, 90 and 120 minute OGTT C peptides. But unlike insulin, fasting and OGTT C peptide groups could not be partitioned according to family history of type 2 diabetes in parents. The difficulty in partitioning C peptide may be due to confounding of increased insulin secretion from insulin resistance by the decreased insulin secretion from beta cell dysfunction, resulting in absence of increase in C peptide in participants with FH.

4.2. Introduction

Insulin action on target tissues and insulin secretion by beta cells of pancreas are two fundamental mechanisms altered in type 2 diabetes (Khan, 2003;

Gerich, 2003; Weyer et al, 1999). Hepatic insulin uptake (Pivovarova et al, 2013), insulin resistance, insulin secretion (Khan, 2003; Weyer et al, 1999) and rate of insulin degradation (Pivovarova et al, 2016) contribute to circulating insulin levels. It was earlier reported that family history of type 2 diabetes caused hyperinsulinemia (Haffner et al.1988), thereby implying genetic and familial contributions to hyperinsulinemia. Genetic factors also contribute to heritability of insulin secretory deficiency by beta cells of pancreas (Elbein et al.1999; Stumvoll et al, 2002) and metabolic changes (Tripathy et al, 2003), resulting in higher prevalence of diabetes in families (Valdez et al, 2007) and among twins (Poulsen et al, 1999).

Morning fasting glucose homeostasis is a steady state condition without diurnal influences and C peptide represents insulin secretion (Khan, 2003; Gerich, 2003; Weyer et al, 1999). Fasting insulin correlated with post glucose load (30, 60 and 120 minute) insulin of oral glucose tolerance test (OGTT). Of these, fasting, 60 and 120 minute OGTT insulin could be partitioned according to family history of type 2 diabetes (FH). The most effective partitioning was possible for fasting insulin (chapter 2). These results indicated that hyperinsulinemia of the fasting state was related to hyperinsulinemia in the 30, 60, 90 and 120 minute OGTT insulin. Though fasting insulin did significantly correlate with 30 minute OGTT insulin, the latter could not be partitioned according to FH. The 30 minute OGTT insulin is the most important phase of insulin secretion.

Clinical interpretation of circulating insulin levels as a predictive marker for type 2 diabetes, for risk calculations of type 2 diabetes mellitus and for evaluation of insulin resistance or insulin secretion have not been successful (Statenet al.2010; Lenzen et al.2006). One of the major reasons for the difficulty in the interpretation is the confounding of hyperinsulinemia by insulin secretory deficiency. The multiple factors that cause hyperinsulinemia increase circulating insulin levels and pancreatic beta cell insulin secretory deficiency decreased circulating insulin levels (Brown, 2003; Robertson, 2004; Prentki & Nolan, 2006). The multiple factors have varying degrees of influence on type 2 diabetes mellitus.

Also, increase in insulin resistance or decreased insulin sensitivity is compensated by increased insulin secretion (Brown, 2003; Prentki & Nolan, 2006)

and is responsible for the negative hyperbolic relation between insulin sensitivity and insulin secretion (Khan, 2003). But insulin secretory deficiency opposed hyperinsulinemia and confounded it.

C peptide may be considered as the secretory component of insulinemia, contributing to hyperinsulinemia. Therefore, C peptide is increased by insulin resistance and decreased by the beta cell dysfunction for insulin secretion (Cauter et al, 1992). It was shown earlier that hyperinsulinemia could be partitioned according to FH (Haffner et al,1988). In this study, the relationship of fasting C peptide with fasting insulin and with 30, 60 and 120 minute OGTT C peptides were analysed. The influences of FH on fasting and OGTT C peptide levels were also studied.

4.3. Aim of study in this section:

Correlation of fasting C peptide with fasting insulin and with OGTT C peptide in young adults. Partitioning of fasting C peptide and OGTT C peptide according to family history of type 2 diabetes in parents.

4.4. Materials and Methods

Healthy participants (n = 80) of Central Kerala state, South India between 18 and 25 years of age, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala from rural population.

Study was approved by the Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011). Informed written consent was obtained from each participant.

4.4.1. Selection of participants, Inclusion and Exclusion criteria

Please refer section 2.2.for selection of participants, and for Inclusion and Exclusion criteriain Chapter 2.

4.4.2.Sample collection

Please refer2.3.forsample collection in Chapter 2.

4.4.3. Diagnostic criteria

Please refer 2.18.for diagnostic criteriainChapter 2.

4.4.4. Statistical Analysis

Please refer 2.19. for statistical analysis inChapter 2

4.4.5. Analytical measurements of C peptide

Please refersection 2.9. forAssay of C peptidein Chapter 2.

4.5. Results

Characteristics of some common C peptide, OGTT and diabetes-related quantitative variables in the total sample (n = 80) are given (Table 4.1). Mean of all the quantitative variables were within their healthy reference intervals. Ranges of all quantitative variables were also within the cut off levels permitted by the inclusion/exclusion criteria. Distributions of the type 2 diabetes related fasting C peptide in the serum samples were positively skewed (Fig. 4.1A and B) with three outliers (Fig. 4.1C). Total sample fasting C peptide histogram also showed a bimodal distribution, probably indicating that the sample could be partitioned (Fig. 4.1A). Log10 transformation converted the C peptide distribution Gaussian as seen by histogram, Q-Q plot and Box Whisker plot (Fig. 4.2). There was again a bimodal distribution (Fig. 4.2A) and there were no outliers (Fig. 4.2C).

Correlation of fasting C peptide with OGTT C peptide or with fasting insulin and triglycerides

Visual examination of the X-Y scatter diagram and calculation of the correlation coefficient of fasting C peptide showed a significant and good positive correlation with 30 minute ($r = 0.584$; $P = <0.001$) and 60 minute ($r = 0.441$; $P < 0.001$) post glucose load OGTT (Fig. 4.3. and Table 4.3.). There was a moderate correlation of fasting C peptide with 90 ($r = 0.279$; $P = 0.012$) and 120 minute C peptide ($r = 0.248$; $P = 0.027$). As C peptide represents insulin secretion, these results indicated that when fasting insulin secretion increased, post glucose load OGTT insulin secretion also increased. Also, the maximum correlation was with 30 minute C peptide and progressively decreased at 60 and 120 minute.

The relationship of type 2 diabetes related parameters, fasting C peptide, fasting insulin and fasting triglycerides, with each other were also examined (Table 4.3). There was a very good and significant correlation of fasting C peptide with fasting insulin ($r = 0.648$; $P < 0.001$) and with fasting triglycerides ($r = 0.409$; $P < 0.001$). Fasting triglycerides also correlated well with fasting insulin ($r = 0.390$; $P < 0.001$). There were direct and significant relationships between fasting type 2 diabetes related parameters, insulin (fasting hyperinsulinemia), C peptide (insulin secretion) and triglycerides (insulin resistance).

Influence of family history of type 2 diabetes on the correlations of fasting C peptide

The above correlations were also examined separately in the groups without and with family history of type 2 diabetes (Table 4.4). There were good and significant correlations of fasting C peptide with 30 minute OGTT C peptide in both groups without and with family history of diabetes. But 60, 90 and 120 minute fasting C peptide showed a significant correlation in the group without family history of type 2 diabetes, but no correlation in the group with family history. These results indicated that in the group with family history of type 2 diabetes, increase in insulin secretion at 60, 90 and 120 minute was confounded by insulin secretory dysfunction in the group with family history of type 2 diabetes mellitus.

The fasting hyperinsulinemia and insulin resistance related parameters fasting insulin, triglycerides and C peptide were analysed for their correlation with each other (Table 4.4). Fasting insulin and fasting C peptide correlated well in both the groups without ($r = 0.601$; $P < 0.001$) and with ($r = 0.710$; $P = < 0.001$) family history of type 2 diabetes. But fasting triglyceride correlated better with fasting insulin and fasting C peptide in the groups with family history of type 2 diabetes than in the group without family history. These results indicated that triacylglycerol, which is related to insulin resistance, increased more with family history of type 2 diabetes.

Family history of type 2 diabetes could not be used for partitioning fasting and OGTT C peptides

The above results on correlation showed differences in the relationship of family history of diabetes with insulin resistance and insulin secretion parameters. Therefore, various groups of C peptide were compared in groups without and with family history of type 2 diabetes (Table 4. 5). It was observed that there was no significant difference of mean fasting C peptide in the groups without and with family history of diabetes in fasting, and in 30 minute, 60 minute, 90 minute and 120 minute C peptide.

Evaluation of the 95% confidence intervals of fasting C peptide was done in the groups with and without family history of type 2 diabetes mellitus. Comparison of the 95% CI of mean fasting C peptide concentrations in groups without and with family history of type 2 diabetes mellitus showed overlapping mean C peptide confidence interval in both groups (Table 4.6 and Fig. 4.4). Similarly, mean post glucose load OGTT C peptide also did not show any difference in the 30, 60, 90 and 120 minute OGTT C peptide (Table 4.6 and Fig. 4.5). These surprising results indicated that increase in insulin secretion resulting from insulin resistance may be confounded by decrease in insulin secretion resulting from beta cell dysfunction.

4.6. Discussion

It was earlier observed that fasting hyperinsulinemia is influenced by family history of type 2 diabetes (Haffner et al,1988) and that both fasting and 60 minute and 120 minute post glucose load OGTT insulin could be partitioned according to family history of type 2 diabetes mellitus (Chapter 3). It was also observed that fasting insulin correlated with post glucose load OGTT insulin and that fasting hyperinsulinemia could be better partitioned than post glucose load insulin. Fasting C peptide is the secretary component of fasting insulinemia or fasting hyperinsulinemia. Insulin resistance is a component and contributes to fasting hyperinsulinemia and increased insulin secretion. Therefore, it was assumed that the secretary component of hyperinsulinemia, C peptide, may also be partitioned according to family history of type 2 diabetes. If so, then fasting C peptide would also correlate with post glucose load C peptide.

The observed results of this study showed that fasting C peptide did correlate well with post glucose OGTT C peptide (Table 4.3) but there was a major difference. The correlation of fasting C peptide was better with 30 minute than 60, 90 and 120 minute OGTT C peptides (Table 4.3). On the other hand, the correlation of fasting insulin was better with 60 and 120 minute OGTT insulin than with 30 minute insulin (Chapter 3). It is important to note that 30 minute C peptide is the major secretary component. The 90 and 120 minute control of blood glucose by insulin is important as a risk factor detrimental to health and for mortality (Meigs et al, 2003; Sorkin et al, 2005; The DECODE study group, 1999).

The correlation of fasting C peptide with OGTT C peptide was also examined after partitioning the sample into groups without and with family history of type 2 diabetes mellitus (Table 4.4). As 30 minute C peptide represents the most important secretory phase of insulin, the correlation with fasting C peptide was maximum in the group with and without family history of type 2 diabetes. There was least correlation of fasting C peptide with 120 minute C peptide in both the groups. Decrease in insulin secretion confounding increase in secretion from insulin resistance should be seen maximum in the group with family history of type 2 diabetes. This argument may explain the lack of correlation of 60 and 120 minute C peptide with fasting C peptide (Table 4.4). As insulin resistance was high in the group with family history of type 2 diabetes, both fasting C peptide and fasting insulin correlated well with fasting triglycerides in that group. But correlated poorly, in the group without family history of type 2 diabetes.

This study also showed that neither fasting C peptide nor 30, 60, 90 and 120 minute C peptide could be partitioned according to family history of type 2 diabetes mellitus (Table 4.5, 4.6; Fig. 4.4. and 4.5). This result was despite the fact that fasting C peptide correlated well with fasting insulin and post glucose load C peptides (Table 4.3). It has been reported earlier that insulin secretion has a genetic component and is decreased in individuals with family history of type 2 diabetes. Therefore, the reason for the lack of significant difference in fasting and post glucose load OGTT may be the confounding of increased insulin secretion from insulin resistance by decreased insulin secretion from beta cell dysfunction.

To conclude, insulin secretion increased with insulin resistance. Therefore, fasting and post glucose load insulin secretion should increase during insulin resistance and correlate. The earlier phase of insulin secretion was correlating better with fasting insulin secretion. But there was no correlation of fasting C peptide with 60, 90 and 120 minute OGTT C peptide in the group with family history of type 2 diabetes. This lack of correlation may attributed to confounding by beta cell dysfunction. But the most significant finding was that family history of type 2 diabetes could not be used for partitioning fasting C peptide, unlike fasting insulin which could be partitioned. This observation was despite the fact that fasting C peptide correlated well with fasting insulin.

Table 4.1. Characteristics of C peptide, OGTT and type 2 diabetes mellitus-related parameters in the total sample of healthy young adults, males and females, with and without family history of type 2 diabetes mellitus in parents (n = 80). OGTT is oral glucose tolerance test.

Quantitative variables n = 80 (Standard Units) (Conventional Units)	**Mean±SD in Standard Units (Conventional Units)**	**Range in Standard Units (Conventional Units)**
Age (years)	(21.80±1.91)	(18 – 25)
BMI (kg/m^2)	(21.54±3.43)	(15.47 – 29.09)
Waist circumference (cm)	(78.38±7.89)	(64.50 – 99.00)
Fasting Glucose (mmol/L) (mg/dL)	4.89±0.523 (88.07±9.43)	3.97 - 6.70 (71.50 – 120.70)
2 hour OGTT, Glucose (mmol/L) (mg/dL)	5.47±1.34 (98.55±24.11)	2.77 – 9.76 (49.91 – 175.80)
Fasting Insulin (pmol/L) (µIU/ml)	37.16±18.37 (6.19±3.06)	12.24 – 106.14 (2.04 – 17.69)
Fasting C peptide (nmol/L) (ng/mL)	0.596±0.191 (1.79±0.575)	0.33 – 1.19 (0.98 - 3.57)
30 minute C peptide (nmol/L) (ng/mL)	2.44±0.842 (7.32±2.53)	1.00 – 5.66 (3.01– 17)
60 minute C peptide (nmol/L)) (ng/mL)	2.79±0.886 (8.38±2.66)	1.33 – 5.19 (3.98 – 15.60)
90 minute C peptide (nmol/L) (ng/mL)	2.52±0.994 (7.56±2.98)	0.94 – 5.83 (2.81 – 17.50)
120 minute C peptide (nmol/L)) (ng/mL)	2.22±1.03 (6.67±3.08)	0.47 – 5.73 (1.41 – 17.20)
Total Cholesterol (mmol/L) (mg/dL)	4.62 ±0.820 (178.51±31.68)	3.42 - 6.89 (132 – 266.00)
Triglycerides (mmol/L) (mg/dL)	0.893±0.396 (79.00±35.06)	0.42 – 2.72 (37 – 241)
LDL Cholesterol (mmol/L) (mg/dL)	2.87±0.735 (110.86±28.36)	1.48 – 5.05 (57 - 195)
HDL Cholesterol (mmol/L) (mg/dL)	1.34±0.360 (51.61±13.89)	0.65 – 2.25 (25 – 87)

Table 4.2. Distribution of fasting, 30, 60, 90 and 120 minute post glucose load OGTT C peptide before and after log_{10} transformation in the total sample (n = 80).

OGTT C Peptide (n = 80)	**(before log_{10} transformation) (nmol/L)**		**(after log_{10} transformation)**	
	Kolmogorov-Smirnova P	**Shapiro Wilk test P**	**Kolmogorov-Smirnova P**	**Shapiro Wilk test P**
Fasting	0.095	<0.001	0.200	0.125
30 minute	0.200	0.009	0.200	0.689
60 minute	0.077	0.017	0.200	0.774
90 minute	0.200	0.020	0.021	0.127
120 minute	0.200	0.003	0.174	0.075

Fig. 4.1. Distribution of fasting C peptide data in the total sample. A: Histogram, B: Q-Q plot, C: Box Whisker plot.

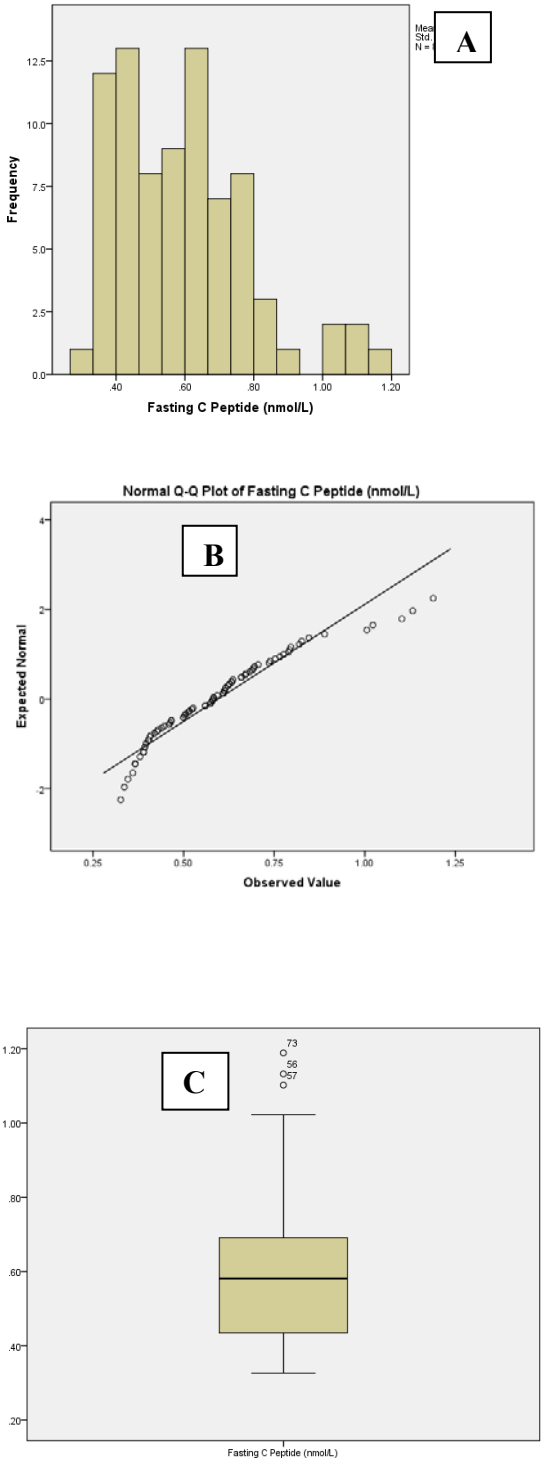
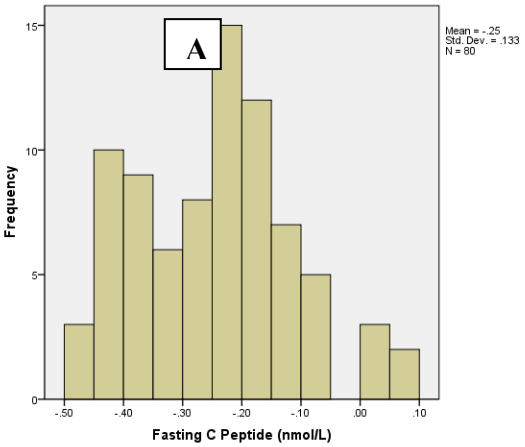
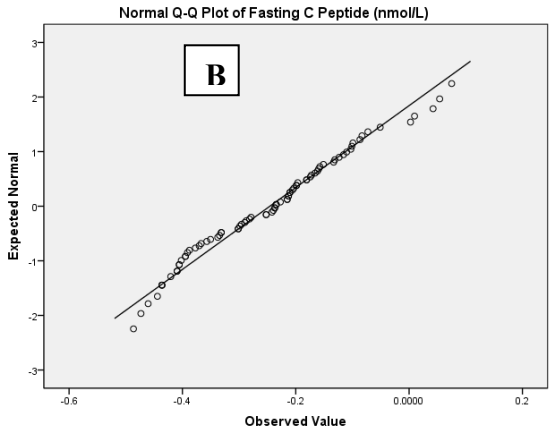


Fig. 4.2. Distribution of fasting C peptide after $\log_{10}$ transformation of the total sample: A. Histogram, B: Q-Q plot, C: Box Whisker plot.





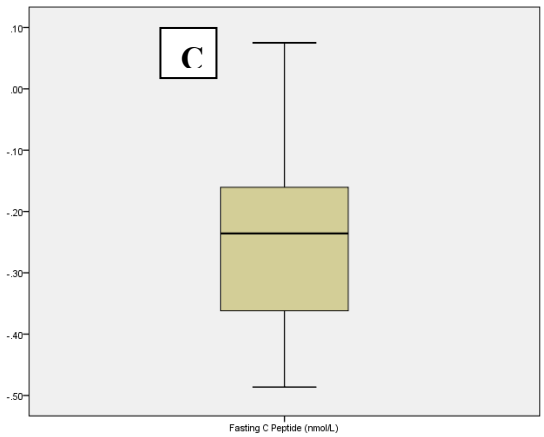


Table 4.3. Pearson's correlation of fasting C peptide (Row No. 1) with 30, 60, 90 and 120 minute OGTT C peptide or with fasting insulin and triglyceride (Rows Nos. 2 – 7) or between fasting insulin and triglyceride (Row 8) in the total sample. All quantitative variables were log_{10} transformed. At least one of the two correlating quantitative variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

Row No.	Quantitative variables (n = 80)	95% Confidence Interval of mean	Shapiro Wilk test (after log_{10} transformation) P	Correlation coefficient (r)	Significance of correlation P
1.	**Fasting C peptide (nmol/L)**	0.553 – 0.638	0.125	--	--
2.	**30 minute C peptide (nmol/L)**	2.25 – 2.62	0.689	0.584	<0.001
3.	**60 minute C peptide (nmol/L)**	2.59 – 2.99	0.774	0.441	<0.001
4.	**90 minute C peptide (nmol/L)**	2.30 – 2.74	0.127	0.279	0.012
5.	**120 minute C peptide (nmol/L)**	1.99 – 2.45	0.075	0.248	0.027
6.	**Fasting insulin (pmol/l)**	33.08 – 41.25	0.171	0.648	<0.001
7.	**Triglyceride (mmol/l)**	0.805 – 0.981	0.013	0.409	<0.001
8.	**Fasting insulin vs Triglyceride**	-	-	0.390	<0.001

Fig. 4.3. X-Y scatter diagram of fasting C peptide (0 minute) with 30 (A), 60 (B) 90 (C) or120 (D) minute post glucose load OGTT C peptide levels in the total sample.

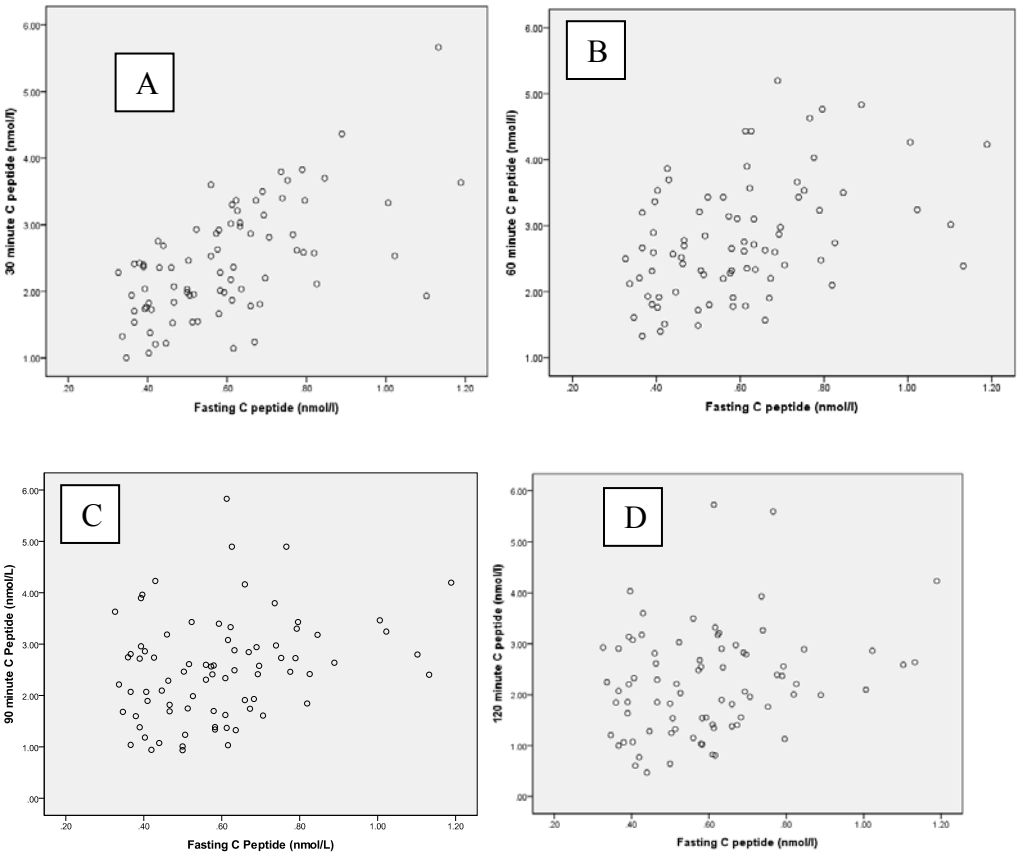


Table 4.4. Pearson's correlation of fasting C peptide (Row No. 1) with 30, 60,90 and 120 minute OGTT C peptide or with fasting insulin and triglyceride (Rows Nos. 2 – 7) or between fasting insulin and triglyceride (Row No. 8) in the groups without and with family history of type 2 diabetes in parents (FH). At least one of the two correlating quantitative variables should have Gaussian distribution by Shapiro Wilk test.

Row No.	Quantitative variables (n = 80)	Groups without and with FH (correlation calculated after log_{10} transformation)					
		Without FH (n = 51)			With FH (n = 29)		
		Shapiro-Wilk test P	Correlation coefficient (r)	Significance of correlation P	Shapiro Wilk test P	Correlation coefficient (r)	Significance of correlation P
1.	**Fasting C peptide (nmol/L)**	0.619	--	--	0.174	--	--
2.	**30 minute C peptide (nmol/L)**	0.532	0.595	<0.001	0.878	0.576	0.001
3.	**60 minute C peptide (nmol/L)**	0.638	0.556	<0.001	0.757	0.286	0.132
4.	**90 minute C peptide (nmol/L)**	0.545	0.317	0.023	0.226	0.227	0.236
5.	**120 minute C peptide (nmol/L)**	0.632	0.279	0.047	0.139	0.206	0.283
6.	**Fasting Insulin (pmol/l)**	0.591	0.601	<0.001	0.250	0.710	<0.001
7.	**Triglyceride (mmol/l)**	0.004	0.296	0.035	0.811	0.553	0.002
8.	**Fasting Insulin vs Triglyceride**	-	0.195	0.170	--	0.590	0.001

Table 4.5. Comparison of fasting C peptide in groups without and with family history of type 2 diabetes mellitus in parents (FH). As groups were positively skewed, log_{10} transformation was done, followed by Student's t test for Gaussian distributed insulin data or the non parametric Mann Whitney U test. %CV is percent coefficient of variation.

Comparison of groups without and with FH								
Fasting and OGTT C peptide (pmol/L)	**WithoutFH** (n = 51)		**With FH** (n = 29)		**C peptide (after log_{10} transformation)**			
	Mean±SD (Range)	**%CV**	**Mean ±SD (Range)**	**%CV**	**Shapiro-Wilk test for normality P**		**Levene's test for equality of variances P**	**Student t-test P**
					Without FH	With FH		
Fasting C peptide	0.584±0.163 (0.33 – 1.02)	27.91	0.617±0.235 (0.34 – 1.19)	38.09	0.619	0.174	0.062	0.696
30 minute C peptide	2.42±0.787 (1.00 – 4.36)	32.52	2.47±0.945 (1.07 – 5.66)	38.26	0.532	0.878	0.803	0.907
60 minute C peptide	2.77±0.868 (1.33 – 4.83)	31.34	2.84±0.930 (1.51 – 5.19)	32.75	0.638	0.757	0.665	0.736
90 minute C peptide	2.47±1.011 (0.94 – 5.83)	40.93	2.59±0.976 (0.94 – 4.90)	37.68	0.545	0.226	0.756	0.586
120 minute C peptide	2.17±1.14 (0.47 – 5.73)	52.53	2.31±0.805 (0.77 – 4.23)	34.85	0.632	0.139	0.081	0.257

Table 4.6. Comparison of 95% CI of mean C peptide concentrations in groups without and with family history of type 2 diabetes mellitus.

Fasting and OGTT serum C Peptide (pmol/L)	95% CI of mean	
	Without family history	With family history
Fasting	0.54 – 0.630	0.53 – 0.706
30 minute	2.20 – 2.64	2.11 – 2.83
60 minute	2.52 – 3.01	2.48 – 3.19
90 minute	2.19 – 2.76	2.22 – 2.96
120 minute	1.85 – 2.49	2.01 – 2.62

Fig. 4.4. Comparison of 95% confidence intervals of mean fasting C peptide in groups without and with family history of type 2 diabetes in parents.

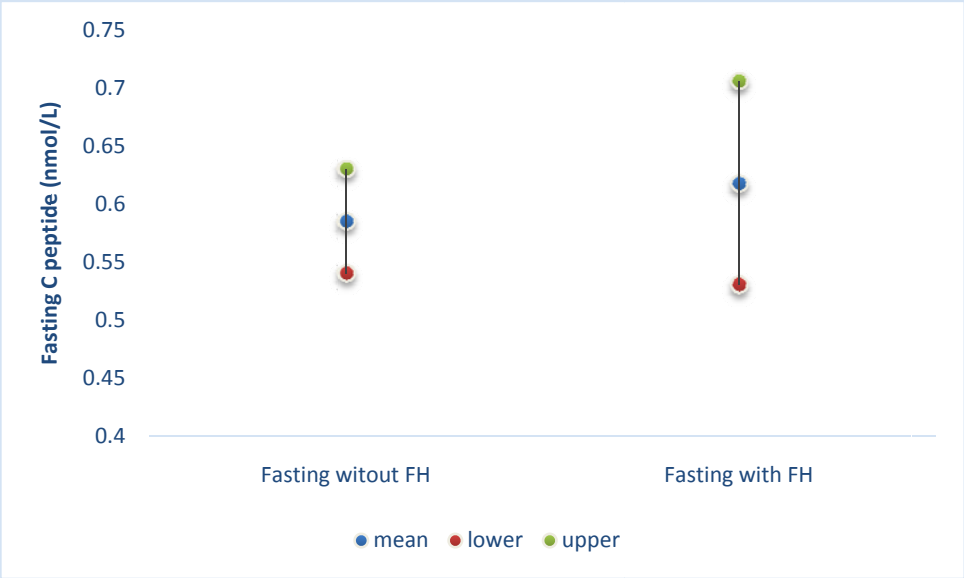
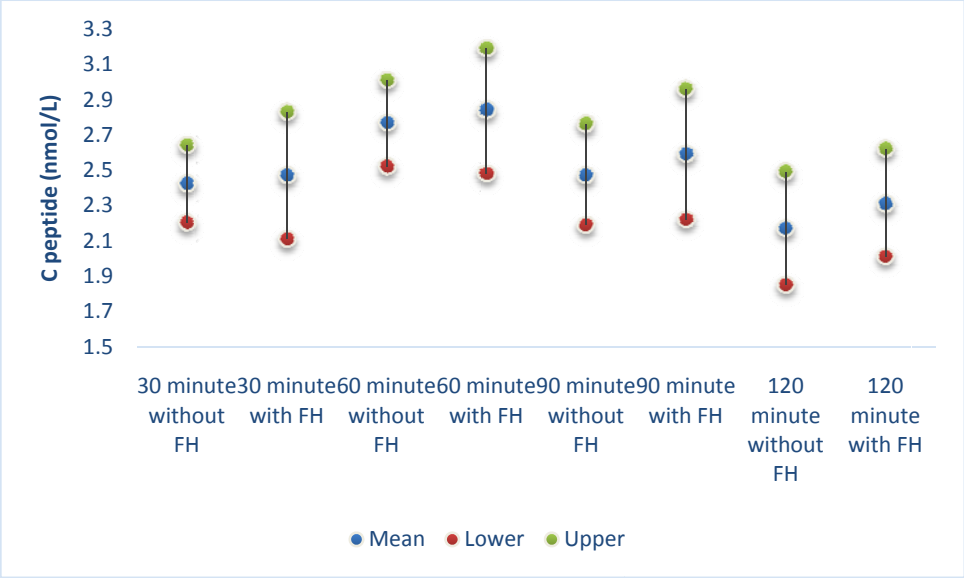


Fig. 4.5. Comparison of 95% confidence intervals of mean of 30, 60, 90 and 120 minute OGTT C peptide from sample of participants without and with family history of type 2 diabetes in parents.



Chapter 5

Gender differences in fasting and post OGTT C peptide in young adults with and without family history of type 2 diabetes mellitus

5.1. Abstract

Premenopausal women with regular menstrual cycles have enhanced insulin sensitivity when compared to adult men and was proposed to be the cause of reduced incidence of type 2 diabetes mellitus in premenopausal women. The lower insulin resistance can reduce insulin secretion and circulating insulin levels. Insulin secretion may be represented by the circulating levels of C peptide. In this study, the correlation between fasting and with oral glucose tolerance test (OGTT) C peptide, and the gender differences in the mean C peptide after partiting were analysed. Fasting and oral glucose tolerance test C peptide, and fasting insulin and triglycerides were assayed in young individuals partitioned into males and females, without and with family history of type 2 diabetes mellitus. C peptide and type 2 diabetes mellitus-related parameters were also assayed in the fasting sample. The cut-off levels of the inclusion of some these parameters were kept higher to include the effects of insulin resistance. There were similarities in the correlation of fasting C peptide with 30 and 60 minute OGTT C peptide and fasting insulin. But there were strong gender differences in the correlation of fasting C peptide with 90 and 120 minute OGTT C peptide and fasting triglycerides. When gender differences, in the mean fasting C peptide, insulin, triglycerides were evaluated, all the three parameters were increased in the male sample with family history of type 2 diabetes. Gender differences in the correlations of fasting C peptides with OGTT C peptides and with fasting insulin and triglycerides, and the gender differences in the mean C peptides of these groups were required for understanding the future risk of hyperinsulinemia and in the clinical interpretation of fasting C peptide levels.

5.2. Introduction

The genetic factors have lead to clustering and increased prevalence of type 2 diabetes and impaired glucose tolerance in families (Gottlieb, 1980; Li H, Isomaa et al, 2000; Hilding et al, 2006), its heritability (Almgren et al, 2011; Poulsen et al, 1999) and the high degree of concordance of in monozygotic twins (Poulsen et al, 1999; Kyvik et al, 1995). There are racial and ethnic differences in the prevalence, insulin sensitivity, insulin response and risk of diabetes. There are also differences, with maternal and paternal history of diabetes, in the prevalence of diabetes and

impaired glucose tolerance in offspring (Kodama et al, 2013; Mitchell et al, 1993; Valdez et al, 2007). There is correlation between prevalence of type 2 diabetes and genetic admixture in hybrid populations (Serjeantson et al, 1983; Brosseau et al, 1979).

Gender differences in the prevalence of diabetes with paternal and maternal familiality of diabetes in Mexican Americans and non Hispanic whites from the San Antonio Heart Study, and in participants who are primarily Caucasians in the Framingham Offspring Study had been reported earlier (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000). The Framingham Offspring Study showed that offspring with maternal diabetes and an age of onset of less than 50 years had increased risk of diabetes (Meigs et al, 2000).

The prevalence of diabetes is higher among the offspring of diabetic mothers than among the offspring of non diabetic mothers or diabetic fathers. These observations have been made in clinical and experimental studies (Meigs et al, 2000; Kasperska-Czyzyk et al, 1996; Pettitt et al, 1988; Thomas et al,1994; Han et al, 2007). Exposure in utero to a diabetic mother is associated with a higher risk of obesity, IGT and diabetes in offspring (Pettitt et al, 1988; Dabelea et al, 2000; Silverman et al, 1995). Insulin secretion has been shown to be abnormal in offspring of diabetic mothers (Aerts et al, 1988).

Familiality of diabetes may be due to genetic and non genetic factors (Dabelea et al, 2000; Han et al, 2007; Doria, 2008). Non genetic factors may be epigenetic factors or non epigenetic issues such as cultural, behavioural and environmental aspects specific to the family. Familiality may also result from intrauterine influences on fetal growth and development, which can lead to changes later in life (Dabelea et al, 2000; Silverman et al,1995; Aerts et al,1988). The intrauterine effects and mitochondrial inheritance may also contribute to maternal transmission of diabetes.

5.3. Aim of Study in this Section

In this study, the correlation between fasting C peptide with oral glucose tolerance test (OGTT) C peptide, and the gender differences in the mean C peptide after partitioning were analysed.

5.4. Materials and Methods

Healthy participants (n = 80) of Central Kerala state, South India between 18 and 25 years of age, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala from rural population.

Study was approved by the Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011). Informed written consent was obtained from each participant.

5.4.1. Selection of participants, Inclusion and Exclusion criteria

Please refer section 2.2 Selection of participants, Inclusion and Exclusion criteriain chapter 2.

5.4.2.Sample collection

Please refer2.3Sample collections in chapter 2.

5.4.3. Diagnostic criteria

Please refer 2.18 Diagnostic criteriainchapter 2

5.4.4. Statistical Analysis

Please refer 2.19 Statistical Analysis inchapter 2

5.4.5. Analytical measurements of C peptide

Please refersection 2.9. Assay of C peptidein chapter 2.

5.5. Results

Characteristics of C peptide and type 2 diabetes-related quantitative variables in males (n = 26) and in females (n = 54) are given (Table 5.1). Mean ±SD and range of these variables were evaluated, and their gender differences were compared. Mean of all the variables were within their expected reference intervals and upper limit of ranges were below the specified cut off levels of inclusion

criteria. Fasting insulin, fasting C peptide and lipid profile parameters, except HDL cholesterol, were higher in males. HDL cholesterol was higher in females.

Pearson's correlation of fasting C peptide was evaluated with other parameters strongly related to type 2 diabetes in the male (n = 26) (Table 5.2 A) and in the female samples (n = 54) (Table 5.2B). This was done to study the gender difference in the correlation of fasting C-peptide and other quantitative variables. There was a good correlation of fasting C peptide with 30 and 60 minute OGTT C peptide ($P < 0.001$ and $P = 0.002$) and with fasting insulin ($P < 0.001$) in both male and female samples (Table 5.2).

But there were strong gender differences in the correlations of fasting C peptide with 90 and 120 minute OGTT C peptide, and with fasting triglycerides. There was a good correlation of fasting C peptide with 90 ($P = 0.004$) and 120 ($P = 0.008$) minute OGTT C peptide, and with fasting triglyceride in the male sample ($P = 0.006$) but not in the female sample. In the female sample, these correlations had significance of $P = 0.150$, $P = 0.156$ and $P = 0.0143$, respectively.

To demonstrate the differences in the correlations in the male and in the female sample X-Y scatter plot of fasting C peptide and fasting triglyceride were made to appreciate the differences. In the male sample, the scatter diagram showed a direct and increasing relationship (Fig. 5.1A), but in the female sample the scatter picture showed a horizontal distribution with increasing C peptide, except for two samples (Fig. 5.1B).

The correlation of fasting C peptide was also analysed separately in the male sample (n = 15) (Table 5.3 A) and in the female sample (n = 36) (Table 5.3 B) of the group without family history of type 2 diabetes mellitus. Fasting C peptide sample correlated well in both male and female with 30 and 60 minute OGTT sample. Similarly, fasting C peptide also correlated well with fasting insulin in both male and female samples but there was a strong gender difference in the correlation of the fasting C peptide with fasting triglyceride. The male sample without family history of type 2 diabetes showed a borderline correlation with fasting triglyceride ($r = 0.471$; $P = 0.076$). But there was no correlation in the female sample ($r = 0.111$; $P = 0.520$). The gender difference in the correlation between fasting C peptide and fasting triglyceride were examined visually by X-Y

scatter diagram (Fig. 5.2). The male fasting C peptide showed a direct relationship with fasting triglyceride (Fig. 5.2A), but in the female sample fasting triglyceride did not increase with fasting C peptide except for one sample (Fig. 5.2B).

Similarly, the correlation of fasting C peptide with OGTT C peptide, fasting insulin and fasting triglyceride were studied in the group with family history of type 2 diabetes. Both in the female sample the correlation of fasting C peptide with OGTT C peptide decrease from 30 to 120 minute (Table 5.4). But the correlation is better in the male sample when compared to that in the female sample. Here also fasting C peptide showed a significant correlation with fasting triglyceride in the male sample (P = 0.035) (Table 5.4 A) but not in the female sample (P = 0.149) (Table 5.4B). The X-Y scatter diagram showed a significant and direct correlation in the male sample (Fig. 5.3A), but the direct relationship between fasting C peptide and triglyceride in the female sample was not significant (Fig. 5.3B).

The above result show a difference in the correlation of fasting C peptide with fasting triglycerides in the group with and without family history of diabetes. Therefore, gender difference in the mean fasting C peptide, insulin, triglycerides were evaluated (Table 5.5) after $\log_{10}$ transformation, which converted the distribution to Gaussian and there was equality of variance in the sample. All the three parameters were increased in the male sample with family history of type 2 diabetes (Table 5.5 A). But in the group without family history of diabetes only the male fasting insulin sample showed a boarder increase with fasting c-peptide and fasting triglycerides (Table 5.5 B).

The influence of family history of type 2diabetes was also studied separately in the male group (n = 26) and in the female group (n = 54) (Table 5.6). In the male group fasting insulin was significantly increased with family history of type 2 diabetes (P = 0.041 (Table 5.6 A). But there was no difference in the fasting C peptide and fasting triglycerides with family history of type 2 diabetes. The female sample showed no difference with and without family history of diabetes for all the three parameters (Table 5.6 B).

5.6. Discussion

Premenopausal women with regular menstrual cycles have enhanced insulin sensitivity when compared to adult men (Jarvis et al, 2013; Park et al, 2003; Yki-Jarvinen H,1984) and was proposed to be the cause of reduced incidence of type 2 diabetes mellitus in premenopausal women(Park et al, 2003; Yki-Jarvinen H,1984). After menopause, there is increase in body fat mass, circulating triglycerides, LDL cholesterol, inflammatory markers and free fatty acids (Carr,2003; Pfeilschifter et al, 2002; Sites et al, 2002). These changes seen after menopause coincided with the sudden decreased insulin sensitivity. Similar observations were made after ovariectomy (Jarvis et al, 2013; Yki-Jarvinen H, 1984; Carr, 2003). Mice with global estrogen receptor deficiency developed hepatic insulin resistance and there was decreased insulin suppression of hepatic glucose production during a euglycemic, hyperinsulinemic clamp (Bryzgalova et al, 2006). However, these mice did not show increased insulin resistance in skeletal muscles. The higher levels of estrogen during pregnancy may increase insulin resistance (Jarvis et al, 2013) which may explain gestational diabetes in women who are predisposed to type 2 diabetes mellitus.

There is negative hyperbolic relationship between insulin sensitivity and insulin secretion (Kahn, 2003). Therefore when insulin resistance increase insulin secretion will also increase C peptide represents the secretory component of circulating insulin. As insulin resistance is lower in premenopausal women C peptide level should also be lower. Our results show that all the parameter related to insulin resistance, fasting insulin, fasting c-peptide, fasting triglycerides were significantly higher in males with family history of type 2 diabetes than females (Table 5.5), but these difference were not significant in the without family history of type 2 diabetes. Similarly, 95% confidence interval of mean (Table 5.4) were relatively higher in male sample with family history of type 2 diabetes. These results indicate that there is lower insulin resistance was observed in young female adults with family history of type 2 diabetes leading to lower insulin secretion and lower c-peptide.

When there is increased fasting insulin resistance there is also increased post glucose load OGTT insulin resistance. Therefore, when fasting C peptide is

increased post glucose load C peptide will also increased. These arguments will explain the direct correlation of fasting C peptide with post glucose load c-peptide in males and female, with and without family history of diabetes (Table 5.3, 5.4).

The circulatory level of triglycerides is directly related to insulin resistance. Higher the insulin resistance higher will be the fasting triglycerides levels. Therefore fasting triglycerides was higher in males with family history of diabetes than in females with and without family history of diabetes (Table 5.3).

Fasting triglycerides did not increase with increasing C peptide in the female sample, but it increase with increasing c-peptide in the male sample (Table 5.3; Fig. 5.2). This observation was seen in the group with and without family history of diabetes. These results indicated that the triglycerides was low in female with and without family history of diabetes and was not correlating with fasting C-peptide.

Significant partitioning could be done in fasting C peptide in the group with family history of type 2 diabetes in males and females (Table 5.5).

Table 5.1. Characteristics of type 2 diabetes mellitus-related parameters in males (n = 26) and in females (n = 54). OGTT is oral glucose tolerance test.

Variables	Males (n =26)		Females (n = 54)	
	Mean ±SD in Standard Units (Conventional Unit)	**Range in Standard Units (Conventional Unit)**	**Mean ±SD in Standard Units (Conventional Unit)**	**Range in Standard Units (Conventional Unit)**
Age (years)	21.38±2.50	18 – 25	22.00±1.53	18 – 25
BMI (kg/m^2)	22.58±3.79	16.61 – 29.09	21.04±3.16	15.47 – 28.08
Waist Circum-ference (cm)	81.94±9.72	64.50 – 99.00	76.66±6.24	66.00 – 95.00
Fasting Glucose mmol/L (mg/dl)	5.04±0.405 (90.88±7.30)	4.42 – 6.04 (79.64 – 108.90)	4.81±0.560 (86.72±10.08)	3.97 – 6.70 (71.50 – 120.70)
2 h OGTT Glucose mmol/L (mg/dl)	5.62±1.38 (101.34± 24.83)	3.26 – 9.76 (58.81 – 175.80)	5.39±1.33 (97.20±23.88)	2.77 – 9.45 (49.91 – 170.30)
Fasting Insulin pmol/L (µIU/ml)	48.04±24.55 (8.01±4.09)	17.10 – 106.14 (2.85 – 17.69)	31.93±11.53 (5.32±1.92)	12.24 – 70.92 (2.04 - 11.82)
Fasting C peptide nmol/L (ng/ml)	0.69±0.246 (2.07±0.740)	0.35 -1.19 (1.04 – 3.57)	0.55±0.14 (1.65±0.419)	0.33 – 0.89 (0.98 – 2.67)
30 minute C peptide nmol/L (ng/ml)	2.51±1.06 (7.55±3.17)	1.00 – 5.66 (3.01 – 17.00)	2.40±0.726 (7.21±2.18)	1.07 – 4.36 (3.22 – 13.10)
60 minute C peptide nmol/L (ng/ml)	2.76±0.876 (8.28±2.63)	1.49 – 4.76 (4.46 – 14.30)	2.81±0.899 (8.44±2.70)	1.33 – 5.19 (3.98 – 15.60)
90 minute C peptide nmol/L (ng/ml)	2.30±0.965 (6.92±2.90)	0.94 – 4.20 (2.81 – 12.60)	2.62±1.00 (7.86±3.00)	1.04 – 5.83 (3.12 – 17.50)
120 minute C peptide nmol/L (ng/ml)	1.96±0.959 (5.88±2.88)	0.47 – 4.76 (1.41 – 12.70)	2.35±1.04 (7.06±3.13)	0.61 – 5.73 (1.82 – 17.20)
Total cholesterol mmol/L (mg/dl)	4.88±1.06 (188.58±40.77)	3.44 -6.89 (133.00 – 266.00)	4.50±0.654 (173.67±25.27)	3.42 – 6.27 (132.00 – 242.00)
Triglycerides mmol/L (mg/dl)	1.07±0.495 (94.77±43.81)	0.52 – 2.72 (46.00 – 241.00)	0.807±0.309 (71.41±27.31)	0.42 – 1.89 (37.00 – 167.00)
LDL Cholesterol mmol/L (mg/dl)	3.25±0.935 (125.46±36.12)	1.99 – 5.05 (77.00 – 195.00)	2.69±0.536 (103.83±20.71)	1.48 – 4.04 (57.00 – 156.00)
HDL Cholesterol mmol/L (mg/dl)	1.14±0.296 (44.23±11.44)	0.65 – 2.25 (25.00 -87.00)	1.43±0.354 (55.17±13.65)	0.80 – 2.20 (31.00 – 85.00)

Table 5.2. Gender differences inthe correlation of fasting C peptide (Row No. 1) with other parameters strongly related to type 2 diabetes in the male (n = 26) (**A**) and in the female (n = 54) (**B**) samples. The type 2 diabetes-related parameters were fasting C peptide (Row 1), 30, 60, 90 and 120 minute OGTT C peptide (Rows 2 – 5), fasting insulin (Row 6) and fasting triglyceride (Row 7). All quantitative variables were $\log_{10}$ transformed. At least one of the two correlating variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

A Row No.	Correlating parameters in males and females	Total male (n = 26)			
		95% Confidence Interval of mean	Shapiro Wilk test (after $\log_{10}$ transformation) P	Correlation coefficient (r)	Significance of correlation P
1.	**Fasting C Peptide (nmol/L)**	0.591 – 0.790	0.520	--	--
2.	**30 minute C Peptide (nmol/L)**	2.09 – 2.94	0.535	0.663	<0.001
3.	**60 minute C Peptide (nmol/L)**	2.40 – 3.11	0.789	0.586	0.002
4.	**90 minute C Peptide (nmol/L)**	1.91 – 2.69	0.037	0.547	0.004
5.	**120 minute C Peptide (nmol/L)**	1.57 – 2.35	0.506	0.506	0.008
6.	**Fasting Insulin (pmol/L)**	38.12 – 57.96	0.312	0.638	<0.001
7.	**Fasting Triglyceride (mmol/l)**	0.871 – 1.27	0.604	0.522	0.006

B Row No.	Correlating parameters in males and females	Total female (n = 54)			
		95% Confidence Interval of mean	Shapiro Wilk test (after log_{10} transformation) P	Correlation coefficient (r)	Significance of correlation P
1.	**Fasting C Peptide (nmol/L)**	0.512 – 0.588	0.086	--	--
2.	**30 minute C Peptide (nmol/L)**	2.20 – 2.60	0.418	0.561	<0.001
3.	**60 minute C Peptide (nmol/L)**	2.56 – 3.05	0.903	0.405	0.002
4.	**90 minute C Peptide (nmol/L)**	2.35 – 2.89	0.957	0.199	0.150
5.	**120 minute C Peptide (nmol/L)**	2.07 – 2.63	0.233	0.196	0.156
6.	**Fasting Insulin (pmol/L)**	28.78 -35.07	0.483	0.577	<0.001
7.	**Fasting Triglyceride (mmol/l)**	0.722 – 0.891	0.020	0.202	0.143

Fig. 5.1. X-Yscatter plot of fasting C peptide and fasting triglycerides in the **total sample** of males (A) and females (B). The parameters were correlating in the male sample (r = 0.522; P = 0.006) but were not correlating in females sample (r = 0.202; P = 0.143).

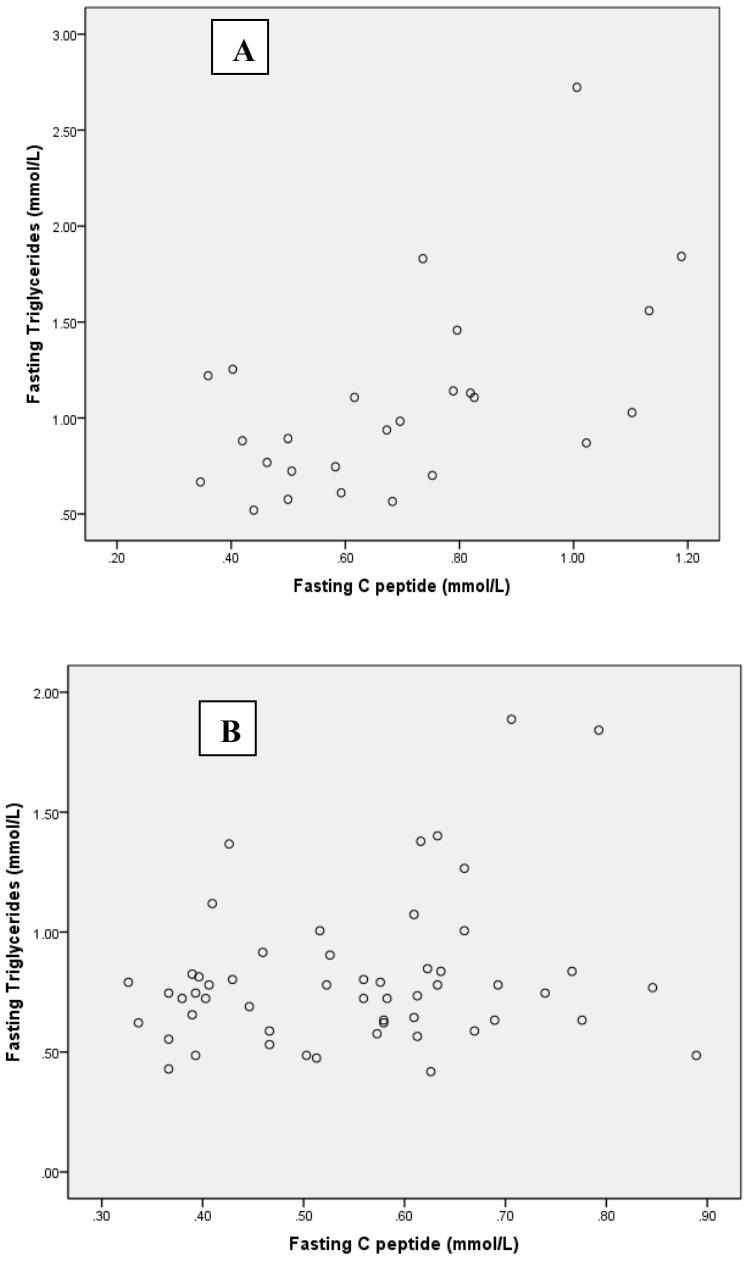


Table 5.3. Gender differences in thecorrelation of fasting C peptide (Row No. 1) with parameters strongly related to type 2 diabetes in the male (n = 15) (A) and female (n = 36) (B) samples of the group **without family history** of type 2 diabetes mellitus (n = 51). The type 2 diabetes-related parameters were fasting C peptide (Row 1), 30, 60, 90 and 120 minute OGTT C peptide (Rows 2 – 5), fasting insulin (Row 6) and fasting triglyceride (Row 7). All quantitative variables were log_{10} transformed. At least one of the two correlating variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

A **Serial No.**	**Correlating parameters in groups without FH**	**Male sample without FH (n = 15)**			
		95% Confidence Interval of mean	**Shapiro Wilk test (after log10 transformation) P**	**Correlation coefficient (r)**	**Significance of correlation P**
Correlation of Fasting C Peptide with other type 2 diabetes-related parameters					
1.	**Fasting C Peptide (nmol/L)**	0.532 – 0.753	0.934	--	--
2.	**30 minute C Peptide (nmol/L)**	1.97 – 3.00	0.204	0.628	0.012
3.	**60 minute C Peptide (nmol/L)**	2.26 – 3.20	0.407	0.610	0.016
4.	**90 minute C Peptide (nmol/L)**	1.64 – 2.76	0.079	0.546	0.035
5.	**120 minute C Peptide (nmol/L)**	1.24 – 2.31	0.961	0.455	0.088
6.	**Fasting Insulin (pmol/L)**	29.70 - 49.97	0.743	0.718	0.003
7.	**Fasting Triglyceride (mmol/l)**	0.685 – 1.33	0.156	0.471	0.076

B Serial No.	Correlating parameters in groups without FH	Female sample without FH (n = 36)			
		95% Confidence Interval of mean	Shapiro Wilk test (after log10 transformation) P	Correlation coefficient (r)	Significance of correlation P
Correlation of Fasting C Peptide with other type 2 diabetes-related parameters					
1.	**Fasting C Peptide (nmol/L)**	0.511 – 0.607	0.564	--	--
2.	**30 minute C Peptide (nmol/L)**	2.14 – 2.64	0.587	0.592	<0.001
3.	**60 minute C Peptide (nmol/L)**	2.48 – 3.08	0.875	0.559	<0.001
4.	**90 minute C Peptide (nmol/L)**	2.25 – 2.93	0.998	0.275	0.104
5.	**120 minute C Peptide (nmol/L)**	1.94 – 2.74	0.791	0.294	0.082
6.	**Fasting Insulin (pmol/L)**	27.49 – 34.10	0.541	0.490	0.002
7.	**Fasting Triglyceride (mmol/l)**	0.701 – 0.894	0.078	0.111	0.520

Fig. 5.2. X-Yscatter plot of fasting C peptide with fasting triglycerides in the male (A) and female (B) sample **without FH**. The parameters had borderline correlation (r = 0.471; P = 0.076) in the male sample but were not correlating (r = 0.111; 0.520) in the female sample.

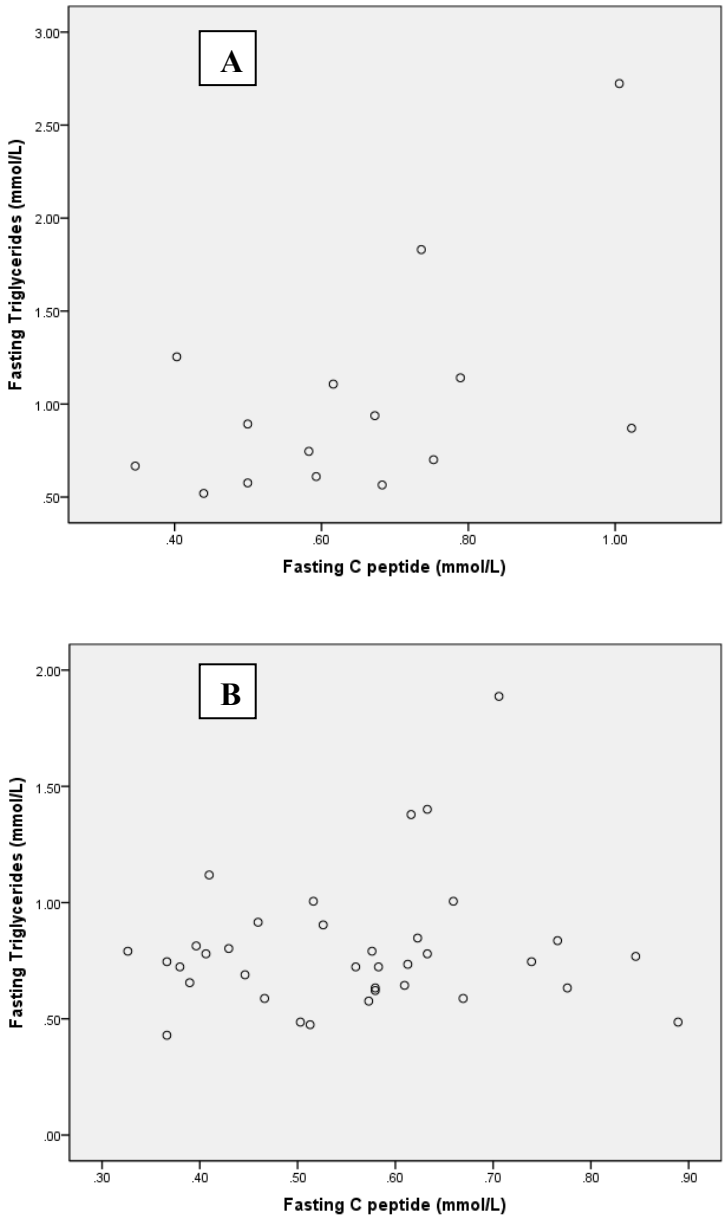


Table 5.4. Gender differences in thecorrelation of fasting C peptide (Row No. 1) with parameters strongly related to type 2 diabetes in the male (n = 11) (A) and in the female (n = 18) (B) samples of the group **with family history** of type 2 diabetes mellitus (n = 29). The type 2 diabetes-related parameters were fasting C peptide (Row 1), 30, 60,90 and 120 minute OGTT C peptide (Rows 2 – 5), fasting insulin (Row 6) and fasting triglyceride (Row 7). All quantitative variables were $\log_{10}$ transformed. At least one of the two correlating variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

A Serial No.	Correlating parameters in group with FH	Male sample with FH (n = 11)			
		95% Confidence Interval of mean	Shapiro Wilk test (after $\log_{10}$ transformation) P	Correlation coefficient (r)	Significance of correlation P
Correlation of Fasting C Peptide with other type 2 diabetes-related parameters					
1.	**Fasting C Peptide (nmol/L)**	0.556 – 0.955	0.329	--	--
2.	**30 minute C Peptide (nmol/L)**	1.71 – 3.40	0.634	0.726	0.011
3.	**60 minute C Peptide (nmol/L)**	2.15 – 3.43	0.650	0.584	0.059
4.	**90 minute C Peptide (nmol/L)**	1.83 – 3.06	0.318	0.552	0.078
5.	**120 minute C Peptide (nmol/L)**	1.59 – 2.84	0.565	0.559	0.074
6.	**Fasting Insulin (pmol/L)**	40.23 – 78.24	0.255	0.548	0.081
7.	**Fasting Triglyceride (mmol/l)**	0.923 – 1.39	0.933	0.638	0.035

B Serial No.	Correlating parameters in group with FH	Female sample with FH (n = 18)			
		95% Confidence Interval of mean	Shapiro Wilk test (after log_{10} transformation) P	Correlation coefficient (r)	Significance of correlation P
Correlation of Fasting C Peptide with other type 2 diabetes-related parameters					
1.	**Fasting C Peptide (nmol/L)**	0.463 – 0.601	0.146	--	--
2.	**30 minute C Peptide (nmol/L)**	2.05 – 2.78	0.385	0.514	0.029
3.	**60 minute C Peptide (nmol/L)**	2.39 – 3.33	0.979	0.118	0.642
4.	**90 minute C Peptide (nmol/L)**	2.17 – 3.19	0.431	0.075	0.766
5.	**120 minute C Peptide (nmol/L)**	2.01 – 2.74	0.086	-0.037	0.883
6.	**Fasting Insulin (pmol/L)**	26.99 – 41.40	0.252	0.747	<0.001
7.	**Fasting Triglyceride (mmol/l)**	0.646 – 1.00	0.487	0.355	0.149

Fig. 5.3. X-Yscatter plot of fasting C peptide with fasting triglycerides in the male (A) and female (B) sample **with FH**. The parameters were correlating in the male sample (r = 0.638; P = 0.035) but were not correlating in the females sample (r = 0.355; P = 0.149).

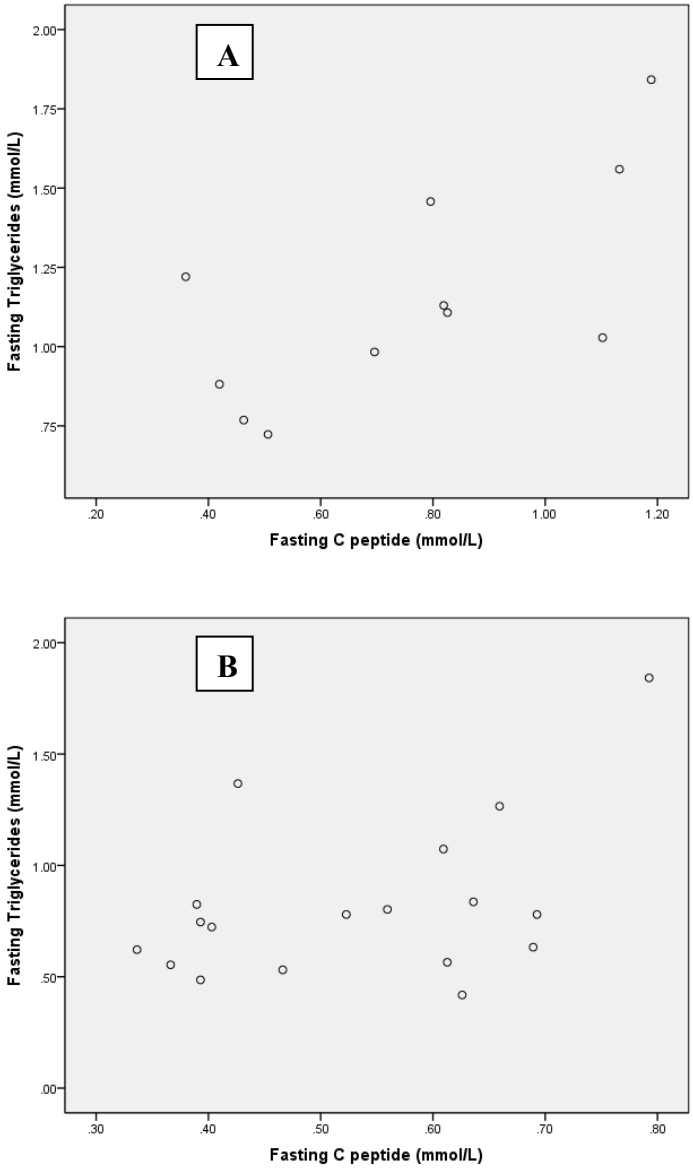


Table 5.5. Gender differences in fasting insulin, fasting C peptide and fasting triglycerides in groups without (n = 51) (A) and with family history (n = 29) (B) of type 2 diabetes mellitus in parents (FH). As groups were positively skewed, log_{10} transformation was done. If the sample had Gaussian distribution and when there was equality of variance parametric Student's t tests were done. Otherwise, non parametric Mann Whitney U tests were done.

Without FH (n = 51)						
A **Variables**	**Male Mean ±SD (Range) (n = 15)**	**Female Mean ±SD (Range) (n = 36)**	**After log_{10} transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Male P	**Female P**		
Fasting Insulin (pmol/L)	39.84±18.29 (17.10 - 80.94)	30.80±9.77 (13.26 - 55.32)	0.743	0.541	0.458	0.053
Fasting C Peptide (nmol/L)	0.643±0.199 (0.35 – 1.02)	0.559±0.141 (0.33 – 0.89)	0.934	0.564	0.485	0.140
Fasting Triglyceride (mmol/l)	1.01±0.586 (0.52 – 2.72)	0.798±0.285 (0.43 – 1.89)	0.156	0.078	0.072	0.128

With FH (n = 29)						
B **Variables**	**Male Mean ±SD (Range) (n = 11)**	**Female Mean ±SD (Range) (n = 18)**	**After log_{10} transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Male P	**Female P**		
Fasting Insulin (pmol/L)	59.23±28.29 (27.84 – 106.14)	34.19±14.48 (12.24 – 70.92)	0.255	0.252	0.475	0.005
Fasting C Peptide (nmol/L)	0.755±0.297 (0.36 – 1.19)	0.532±0.138 (0.34 – 0.79)	0.329	0.146	0.062	0.023
Fasting Triglyceride (mmol/l)	1.15±0.345 (0.72 – 1.84)	0.825±0.359 (0.42 – 1.84)	0.933	0.487	0.498	0.010

Table 5.6. Influence of family history of type 2 diabetes (FH) on fasting insulin, fasting C peptide and fasting triacylglycerols in male (n = 26) and in female (n = 54) groups. As groups were positively skewed, $\log_{10}$ transformation was done. If the sample had Gaussian distribution and when there was equality of variance, parametric tests were done. Otherwise, non parametric Mann Whitney U test was done.

Male (n = 26)						
A **Variables**	**Without FH Mean ±SD (Range) (n = 15)**	**With FH Mean ±SD (Range) (n = 11)**	**After log10 transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Without FH P	**With FH P**		
Fasting Insulin (pmol/L)	39.84±18.29 (17.10 - 80.94)	59.23±28.29 (27.84 – 106.14)	0.743	0.255	0.427	0.041
Fasting C Peptide (nmol/L)	0.643±0.199 (0.35 – 1.02)	0.755±0.297 (0.36 – 1.19)	0.934	0.329	0.187	0.375
Fasting Triglyceride (mmol/l)	1.01±0.586 (0.52 – 2.72)	1.15±0.345 (0.72 – 1.84)	0.156	0.933	0.203	0.199

Female (n = 54)						
B **Variables**	**Without FH Mean ±SD (Range) (n = 36)**	**With Mean ±SD (Range) (n = 18)**	**After log10 transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Without FH P	**With FH P**		
Fasting Insulin (pmol/L)	30.80±9.77 (13.26 – 55.32)	34.19±14.48 (12.24 – 70.92)	0.541	0.252	0.452	0.553
Fasting C Peptide (nmol/L)	0.559±0.141 (0.33 – 0.89)	0.532±0.138 (0.34 – 0.79)	0.564	0.146	0.527	0.496
Fasting Triglyceride (mmol/l)	0.798±0.285 (0.43 – 1.89)	0.825±0.359 (0.42 – 1.84)	0.078	0.487	0.322	0.927

Chapter 6

Gender differences in fasting and post glucose load OGTT insulin in young adults with and without family history of type 2 diabetes mellitus

6.1. Abstract

Gender difference was observed in the fasting and post glucose load oral glucose tolerance test (OGTT) C peptide earlier in this study. The increased insulin secretion in males may be to compensate the increased insulin resistance in males. Increased insulin secretion is one of the factors contributing to hyperinsulinemia. Therefore, in this study the gender difference in the correlation of fasting insulin with OGTT insulin, and the gender difference in the mean fasting insulin groups were analyzed. Fasting and OGTT insulin and fasting triglyceride were assayed in young adult partitioned into males and females, and into groups with and without family history of type 2 diabetes mellitus. It was observed that there was gender difference in the correlation of fasting insulin with OGTT insulin groups. This difference was found to be due to the strong correlation seen in female samples with family history of type 2 diabetes mellitus. The mean fasting insulin and fasting triglyceride were higher in the male samples and in the samples with family history of type 2 diabetes mellitus. It was concluded that there are gender differences in the fasting insulin levels in groups with and without family history of type 2 diabetes mellitus.

6.2. Introduction

Familiality of diabetes may be due to genetic and non genetic factors (Doria et al, 2008; Han et al, 2007; Dabelea et al, 2000). Non genetic factors may be epigenetic factors or non epigenetic issues such as cultural, behavioural and environmental aspects specific to the family. Familiality may also result from intrauterine influences on fetal growth and development, which can lead to changes later in life (Dabelea et al, 2000; Silverman et al, 1995; Aerts et al, 1988). The intrauterine effects and mitochondrial inheritance may also contribute to maternal transmission of diabetes.

Prevalence of diabetes in India showed an increase, as reported from 1988 onwards and was predicted to increase further (Ramachandran et al, 1988; Wild et

al, 2004; Mohan et al, 2007; Sadikot et al, 2004; Whiting et al, 2011). In the year 2000, India had the highest number of individuals with diabetes, and was predicted to increase fastest upto 2030 (Wild et al, 2004; Whiting et al, 2011). Prevalence was higher in urban when compared to rural population (Ramachandran et al, 1988; Mohan et al, 2007; Sadikot et al, 2004; Vijayakumar et al, 2009; Ramachandran et al, 1992; Ramachandran et al, 2001). High prevalence of diabetes was reported in Kerala state, from where this report originates (Vijayakumar et al.2009).

The prevalence of diabetes is higher among the offspring of diabetic mothers than among the offspring of non diabetic mothers or diabetic fathers. These observations have been made in clinical and experimental studies (Meigs et al, 2000; Kasperska-Czyzyk et al, 1996; Pettitt et al, 1988; Thomaset al, 1994; Han et al, 2007). Exposure in utero to a diabetic mother is associated with a higher risk of obesity, IGT and diabetes in offspring (Pettitt et al, 1988; Dabelea et al, 2000; Silverman et al, 1995). Insulin secretion has been shown to be abnormal in offspring of diabetic mothers (Aerts et al, 1988).

Gender differences in the prevalence of diabetes with paternal and maternal familiality of diabetes in Mexican Americans and non Hispanic whites from the San Antonio Heart Study, and in participants who are primarily Caucasians in the Framingham Offspring Study had been reported earlier (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000). The Framingham Offspring Study showed that offspring with maternal diabetes and an age of onset of less than 50 years had increased risk of diabetes (Meigs et al, 2000).

The genetic factors have lead to clustering and increased prevalence of type 2 diabetes and impaired glucose tolerance in families (Gottlieb, 1980; Li et al, 2000; Hilding et al, 2006), its heritability (Almgren et al, 2011; Poulsen et al, 1999) and the high degree of concordance of in monozygotic twins (Poulsen et al, 1999; Kyvik et al, 1995). There are racial and ethnic differences in the prevalence, insulin sensitivity, insulin response and risk of diabetes. There are also differences, with maternal and paternal history of diabetes, in the prevalence of diabetes and impaired glucose tolerance in offspring (Kodama et al, 2013; Mitchell et al, 1993; Valdez et al, 2007). There is correlation between prevalence of type 2 diabetes and

genetic admixture in hybrid populations (Serjeantson et al, 1983; Brosseau et al, 1979).

6.3. Aim of Study in this Section

Evaluation of the gender difference in the correlation of fasting insulin with OGTT insulin and related parameters, and the gender difference in the mean fasting insulin in various groups.

6.4.Materials and Methods

6.4.1. Study Settings and Design

Healthy cross section of participants (n = 90) between 18 and 25 years, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala from rural population of Central Kerala.

The Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011) approved the study. Informed written consent was obtained from each participant aged between 18 and 25 years.

6.4.2. Selection of participants, Inclusion and Exclusion criteria

Please refer 2.2. Selection of participants, Inclusion and Exclusion criteriain Chapter 2.

6.4.3.Sample collection

Please refer2.3.Sample collection in Chapter 2.

6.4.4. Analytical control and insulin measurements

Please refer 2.8. Detailed Description of an Immunochemistry Assay, eg. Insulin Assay in Chapter 2.

6.4.5. Diagnostic criteria

Please refer 2.18. Diagnostic criteriainChapter 2

6.4.6. Statistical Analysis

Please refer 2.19. Statistical Analysis inChapter 2

6.5. Results

Characteristics of insulin and type 2 diabetes-related quantitative variables in males (n = 31) and in females (n = 59) are given (Table 6.1.). Mean±SD and range of these variables were evaluated, and their gender differences were compared. Mean of all the variables are within their expected reference intervals and upper limit of ranges were below the specified cut off levels of inclusion criteria. Distribution of most of the type 2 diabetes related variables in the serum samples were positively skewed. Fasting insulin, fasting triglycerides and LDL cholesterol were higher in males. HDL cholesterol was higher in females.

Pearson's correlation of fasting insulin was evaluated with other parameters strongly related to type 2 diabetes in the male (n = 31) (Table 6.2A) and in the female samples (n = 59) (Table 6.2B). This was done to study the gender difference in the correlation of fasting insulin and other quantitative variables. In the male sample, there was no correlation of fasting insulin with 30, 60 and 120 minute OGTT insulin, except for 90 minute OGTT insulin (P = 0.005) (Table 6.2A). But in the female sample, there a good correlation of fasting insulin with 30, 60, 90 and 120 minute OGTT insulin (Table 6.2B). This was a major difference between male and female with reference to the correlation of fasting insulin. There was no correlation of fasting insulin with fasting triglyceride in both male and female samples.

As there was a gender difference seen in the co-relation of fasting insulin with OGTT insulin, this observation was analyzed further after partitioning the sample into groups without and with family history of type 2 diabetes mellitus. In the male sample without family history of type 2 diabetes mellitus, fasting insulin was not correlated with any other parameters other than 30 minute insulin (Table 6.3A). In the female sample without family history of type 2 diabetes mellitus, fasting insulin was not correlated with any other parameters other than 90 minute

insulin (Table 6.3B). These results showed that in the group without family history of type 2 diabetes mellitus, fasting insulin, both in the male and female samples, did not correlated with OGTT insulin.

Therefore, the group with family history of type 2 diabetes mellitus was partitioned into male and female groups, and also evaluated the correlation of fasting insulin with other insulin values and related parameters. In the male sample, fasting insulin did not correlate with any other parameter (Table 6.4A). But in the female sample fasting insulin correlate significantly with all the OGTT insulin groups and with fasting triaclyglyceride (Table 6.4B). These results showed a strong gender difference in the correlation of fasting insulin in the group with family history of type 2 diabetes mellitus. Therefore, the earlier correlation of fasting insulin with OGTT insulin in the total female sample (Table 6.2B) was due to the correlation in the female sample with family history of type 2 diabetes mellitus.

Gender difference of mean fasting insulin in the groups without and with family history of type 2 diabetes mellitus were evaluated. In the group without family history of type 2 diabetes mellitus the male sample had higher fasting insulin and fasting triacylglyceride than the female sample (Table 6.5A), but only the fasting triacylglyceride was significantly higher. The sample number for fasting insulin was may not be sufficient. The gender difference of fasting insulin was calculated with increased sample number after partitioning in Chapter 7 and 8. But there was no gender difference between male and female mean fasting insulin in the group with family history of type 2 diabetes mellitus (Table 6.5B). These results indicate gender difference in mean fasting insulin and fasting triacylglyceride.

The influence of family history of type 2 diabetes mellitus on males and on females in the samples were evaluated separately (Table 6.6.). In the male group samples were partitioned with and without family history of type2 diabetes mellitus (Table 6.6A). Both fasting insulin and fasting triacylglyceride were higher in the group with family history of type 2 diabetes mellitus, but the increase was not statistically significant. This was due to lower sample number. Therefore,

gender difference and the influence of familiality in fasting insulin was analysed with higher sample number (Chapter 7 & 8).

In the female sample, both fasting insulin and fasting triglyceride were increased in the group with family history of type 2 diabetes mellitus and the increase was significant for fasting insulin ($P<0.001$), and with borderline significant for fasting triglyceride($P = 0.063$) (Table 6.6B).

The 95% confidence interval of mean was compared between fasting insulin in the male and female sample with and without family history of type 2 diabetes mellitus (Fig 6.1A). This analysis was done to further understand the difference in mean fasting insulin. The male samples and samples with family history of type 2 diabetes mellitus had higher fasting insulin than the female samples and the samples without family history of type 2 diabetes mellitus (Fig.6.1A). Increased variations seen in the sample with family history of type 2 diabetes mellitus and in the male sample indicate the requirement of higher sample number.

The fasting triglyceride was higher in male samples but did not show difference in with and without family history of type 2 diabetes mellitus group (Fig 6.1B). Fasting triglyceride was lower in female sample and without family history of type 2 diabetes mellitus sample, when compared with family history of type 2 diabetes mellitus group.

6.6. Discussion

Fasting insulin could be partitioned according to family history of type 2 diabetes mellitus (Chapter 3). Prevalence of type 2 diabetes was higher in the offsprings when there was family history of type 2 diabetes (Meigs et al, 2000; Kasperska-Czyzyk et al, 1996; Pettitt et al, 1988; Thomaset et al, 1994; Han et al, 2007). The genetic factors have lead to clustering of type 2 diabetes in families (Gottlieb, 1980; Li et al, 2000; Hilding et al, 2006), its heritability (Hilding et al 2006; Almgren et al, 2011) and the high degree of concordance of diabetes in monozygotic twins (Poulsen et al, 1999; Kyvik et al, 1995). It was also shown that

there is a strong heritable component in hyperinsulinemia (Haffner et al, 1988) and in beta cell dysfunction. Increase in the prevalence of impaired glucose tolerance and hyperinsulinemia (Gottlieb, 1980; Li et al, 2000; Mitchell et al; 1993; Valdez et al, 2007) in offspring of parents with type 2 diabetes has been reported. Insulin secretion increases when there is insulin resistance (Khan et al, 1993; Khan, 2003). Insulin resistance also contributes to hyperinsulinemia. With this background the effect of gender on hyperinsulinemia was analysed in this study.

Prevalence of type 2 diabetes was reported to higher in offsrings of diabeteic mothers than among non diabeteic mothers and diabetic fathers (Meigs et al, 2000; Kasperska – Czyzyk et al, 1996; Pettitt et al, 1988; Han et al, 2007). Exposure of the fetous in the uterus of a diabetic mother is associated with higher risk of obesity, IGT and diabetes in offspring (Pettitt et al, 1988; Dabelea et al , 2000; Silverman et al, 1995).

Changes in the post menopausal period coincide with sudden decrease in insulin sensitivity. After menopause there is increase in body fat mass, circulating triglycerides, LDL cholesterol, inflammatory markers and free fatty acids (Carr, 2003; Pfeilsehifter et al, 2002; Sites et al, 2002). In premenopausal women with menstrual cycles, there is decrease in insulin resistance when compared to adult men (Jaris et al, 2013; Park et al, 2003; Yki-Jarvinen, 1984).

There was gender difference in the correlation of fasting insulin with post glucose load OGTT insulin. There was a stronger correlation in the female samples with family history of type 2 diabetes. This may be due to increase in beta cell dysfunction in males with family history of type 2 diabetes mellitus. The mean fasting insulin was higher in males than in female samples, and was higher when there was family history of type 2 diabetes. The lower fasting insulin in females may be due to their decreased insulin resistance in females before menopause (Jaris et al, 2013; Park et al, 2003; Yki-Jarvinen, 1984) as the sample population was 18 - 25 years of age. The decreased correlation of fasting insulin in the male samples may be due to the confounding of hyperinsulinemia with insulin secretory deficit which decreases insulin.

The mean fasting triglyceride, which increases in insulin resistance, was also increased along with hyperinsulinemia in males. These results also come from

the gender difference in fasting insulin in males as compared to female samples. Gender differences were also seen in fasting insulin in both groups with and without family history of type 2 diabetes. The gender differences may be attributed to increased insulin sensitivity in premenopausal women and increased beta cell dysfunction in men with family history of type 2 diabetes mellitus.

Table 6.1. Characteristics of type 2 diabetes mellitus-related parameters in males (n = 31) and females (n = 59). OGTT is oral glucose tolerance test.

Quantitative variables (Standard Units) (Conventional Units)	Male (n = 31)		Female (n = 59)	
	Mean ±SD (Conventional Unit)	Range (Conventional Unit)	Mean ±SD (Conventional Unit)	Range (Conventional Unit)
Age (years)	20.77±2.55	18 – 25	22.25±1.52	19 – 25
BMI (kg/m^2)	21.44±3.05	16.54 – 27.11	20.32±2.74	15.47 – 26.67
Waist circum-ference (cm)	73.99±9.17	57.50 – 94	75.93±8.14	59.00 – 92.00
Fasting Glucose (mmol/L) (mg/dl)	5.18±0.511 (93.39±9.22)	4.35 – 6.87 (78.32 – 123.70)	5.14±0.519 (92.67±9.35)	3.97 – 6.37 (71.50 – 114.85)
2 hour OGTT Glucose (mmol/L) (mg/dl)	5.58±1.33 (100.55± 23.95)	2.49 – 9.76 (44.86 – 175.80)	5.72±1.22 (103.14±21.95)	3.84 – 9.45 (69.10 – 170.30)
Fasting Insulin (pmol/L) (µIU/ml)	36.06±17.42 (6.01±2.90)	10.02 – 96.30 (1.67 – 16.05)	33.91±16.92 (5.65±2.82)	8.46 – 97.56 (1.41 – 16.26)
30 minute Insulin (pmol/L) (µIU/ml)	353.44±237.68 (58.91±39.61)	103.20 – 1373.40 (17.20 – 228.90)	397.62±251.91 (66.27±41.98)	69.48 – 1241.76 (11.58 – 206.96)
60 minute Insulin (pmol/L) (µIU/ml)	322.31±205.57 (53.72±34.26)	67.32 – 916.50 (11.22 – 152.75)	351.83±262.76 (58.64±43.79)	85.62 – 1612.08 (14.27 – 268.68)
90 minute Insulin (pmol/L) (µIU/ml)	231.33±215.40 (38.56±35.90)	8.52 – 1053.72 (1.42 – 175.62)	298.51±177.81 (49.75±29.63)	57.60 – 880.86 (9.60 – 146.81)
120 minute Insulin (pmol/L) (µIU/ml)	195.93±142.49 (32.65±23.75)	21.06 – 650.70 (3.51 – 108.45)	239.12±166.35 (39.85±27.72)	24.36 – 880.50 (4.06 – 146.75)
Total cholesterol (mmol/L) (mg/dl)	4.47±780 (172.71±30.13)	2.85 – 6.53 (110.00 – 252.00)	4.65±0.694 (179.38±26.80) **(n = 55)**	3.42 – 6.37 (132.00 – 246.00)
Triglycerides (mmol/L) (mg/dl)	0.931±0.306 (82.35±27.12)	0.47 – 1.57 (42.00 – 139.00)	0.852±0.383 (75.36±33.90) **(n = 55)**	0.42 – 2.51 (37.00 – 222.00)
LDL Cholesterol (mmol/L) (mg/dl)	2.91±0.709 (112.24±27.37)	1.66 – 4.84 (64.00 – 187.00)	2.80±0.639 (108.21±24.69) **(n = 55)**	1.48 – 4.30 (54.00 – 166.00)
HDL Cholesterol (mmol/L) (mg/dl)	1.14±0.174 (44.09±6.71)	0.73 – 1.48 (28.00 -57.00)	1.45±0.323 (55.95±12.48) **(n = 55)**	0.88 – 2.20 (34.00 – 85.00)

Table 6.2. Gender differences inthe Pearson's correlation of fasting insulin (Row No. 1) with other parameters strongly related to type 2 diabetes in the male (n = 31) (A) and in the female (n = 59) (B) samples. The type 2 diabetes-related parameters were fasting insulin (Row 1), 30, 60, 90 and 120 minute OGTT Insulin (Rows 2 – 5), and fasting triglyceride (Rows 6). All quantitative variables were $\log_{10}$ transformed. At least one of the two correlating variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

A **Sl No.**	**Correlating parameters in males and females**	**Total male (n = 31)**			
		95% Confidence Interval of mean	**Shapiro Wilk test (after $\log_{10}$ transformation) P**	**Correlation coefficient (r)**	**Significance of correlation P**
Correlations of Fasting Insulin with other type 2 diabetes -related parameters					
1.	**Fasting Insulin (pmol/L)**	29.67 – 42.45	0.327	-	-
2.	**30 minute Insulin (pmol/L)**	266.26 – 440.62	0.531	0.282	0.124
3.	**60 minute Insulin (pmol/L)**	246.90 – 397.71	0.920	0.205	0.268
4.	**90 minute Insulin (pmol/L)**	152.32 – 310.35	0.100	0.488	0.005
5.	**120 minute Insulin (pmol/L)**	143.66 – 248.19	0.882	0.201	0.278
6.	**Fasting Triglyceride (mmol/l)**	0.818 – 1.04	0.376	0.190	0.307

B Serial No.	Correlating parameters in males and females	Total female (n = 59)			
		95% Confidence Interval of mean	Shapiro Wilk test (after log_{10} transformation P	Correlation coefficient (r)	Significance of correlation P
Correlations of Fasting Insulin with other type 2 diabetes -related parameters					
1.	**Fasting Insulin (pmol/L)**	29.50 - 38.32	0.657	-	-
2.	**30 minute Insulin (pmol/L)**	331.97 – 463.27	0.357	0.270	0.038
3.	**60 minute Insulin (pmol/L)**	283.36 – 420.31	0.466	0.413	0.001
4.	**90 minute Insulin (pmol/L)**	252.17 – 344.85	0.321	0.413	0.001
5.	**120 minute Insulin (pmol/L)**	195.77 – 282.47	0.397	0.349	0.007
6.	**Fasting Triglyceride (mmol/l)**	0.748 – 0.955 **(n = 55)**	0.005	0.231	0.090

Table 6.3. Gender differences in thePearson's correlation of fasting insulin (Row No. 1) with parameters strongly related to type 2 diabetes in male (n = 20) (A) and in female (n = 37) (B) samples of the group without family history of type 2 diabetes mellitus (FH) (n = 57). The type 2 diabetes-related parameters were fasting insulin (Row 1), 30, 60, 90 and 120 minute OGTT Insulin (Rows 2 – 5), and fasting triglyceride (Rows 6). At least one of the two correlating variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

A **Serial No.**	**Correlating parameters in groups without FH**	**Without FH Male (n = 20)**			
		95% Confidence Interval of mean	**Shapiro Wilk test P**	**Correlation coefficient (r)**	**Significance of correlation P**
Correlations of Fasting Insulin with other type 2 diabetes -related parameters					
1.	**Fasting Insulin (pmol/L)**	26.68 – 37.53	0.915	-	-
2.	**30 minute Insulin (pmol/L)**	241.33 – 395.11	0.075	0.442	0.051
3.	**60 minute Insulin (pmol/L)**	202.18 – 337.96	0.257	0.143	0.546
4.	**90 minute Insulin (pmol/L)**	123.99 – 237.18	0.029	0.198	0.402
5.	**120 minute Insulin (pmol/L)**	105.74 – 200.75	0.031	0.077	0.748
6.	**Fasting Triglyceride (mmol/l)**	0.794 – 1.04	0.400	-0.051	0.832

B Serial No.	Correlating parameters in groups without FH	Without Female, (n = 37)			
		95% Confidence Interval of mean	Shapiro Wilk test P	Correlation coefficient (r)	Significance of correlation P
Correlations of Fasting Insulin with other type 2 diabetes -related parameters					
1.	**Fasting Insulin (pmol/L)**	24.31 – 30.70	0.522	-	-
2.	**30 minute Insulin (pmol/L)**	307.52 – 469.90	<0.001	0.125	0.461
3.	**60 minute Insulin (pmol/L)**	235.93 – 338.93	0.002	0.205	0.224
4.	**90 minute Insulin (pmol/L)**	211.63 – 328.28	0.001	0.333	0.044
5.	**120 minute Insulin (pmol/L)**	151.66 – 267.71	<0.001	0.265	0.113
6.	**Fasting Triglyceride (mmol/l)**	0.677 – 0.923	<0.001 **(n = 36)**	-0.212	0.214

Table 6. 4. Gender differences in thePearson's correlation of fasting insulin (Row No. 1) with other parameters strongly related to type 2 diabetes in the male (n = 11) (A) and in the female (n = 22) (B) samples of the group with family history of type 2 diabetes mellitus (FH) (n = 33). The type 2 diabetes-related parameters were fasting insulin (Row 1), 30, 60, 90 and 120 minute OGTT Insulin (Rows 2 – 5), and fasting triglyceride (Rows 6). All quantitative variables were $\log_{10}$ transformed. At least one of the two correlating variables should have Gaussian distribution by Shapiro Wilk test for parametric calculation of correlation.

A **Sl No.**	**Correlating parameters in group with FH**	**With FH Male (n = 11)**			
		95% Confidence Interval of mean	**Shapiro Wilk test (after $\log_{10}$ transformation) P**	**Correlation coefficient (r)**	**Significance of correlation P**
1.	**Fasting Insulin (pmol/L)**	27.23 – 59.23	0.135	-	-
2.	**30 minute Insulin (pmol/L)**	193.43 – 641.50	0.272	0.070	0.837
3.	**60 minute Insulin (pmol/L)**	237.74 – 596.82	0.533	0.105	0.759
4.	**90 minute Insulin (pmol/L)**	114.49 – 532.73	0.825	0.292	0.384
5.	**120 minute Insulin (pmol/L)**	155.11 – 391.94	0.899	0.039	0.910
6.	**Fasting Triglyceride (mmol/l)**	0.696 – 1.21	0.458	0.478	0.137

B Serial No.	Correlating parameters in group with FH	With FH Female, (n = 22)			
		95% Confidence Interval of mean	Shapiro Wilk test (after log10 transformation P	Correlation coefficient (r)	Significance of correlation P
Correlations of Fasting Insulin with other type 2 diabetes -related parameters					
1.	**Fasting Insulin (pmol/L)**	35.39 – 53.98	0.472	- -	- -
2.	**30 minute Insulin (pmol/L)**	292.61 – 532.58	0.987	0.514	0.014
3.	**60 minute Insulin (pmol/L)**	300.16 – 620.13	0.133	0.439	0.041
4.	**90 minute Insulin (pmol/L)**	268.46 – 424.61	0.339	0.423	0.050
5.	**120 minute Insulin (pmol/L)**	225.27 – 351.96	0.623	0.445	0.038
6.	**Fasting Triglyceride (mmol/l)**	0.752 – 1.145 (n = 19)	0.988	0.595	0.007

Table 6. 5. Gender differences in fasting insulin and fasting triglycerides in groups without (n = 57) (A) and with family history (n = 33) (B) of type 2 diabetes mellitus in parents (FH). As groups were positively skewed, log_{10} transformation was done. If the sample had Gaussian distribution and when there was equality of variance parametric Student's t tests were done. Otherwise, non parametric Mann Whitney U tests were done.

Without FH (n = 57)						
A **Variables**	**Male Mean ±SD (Range) (n = 20)**	**Female Mean ±SD (Range) (n = 37)**	**After log10 transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Male P	**Female P**		
Fasting Insulin (pmol/L)	32.11±11.59 (10.02 – 54.48)	27.51±9.59 (13.26 – 55.32)	0.187	0.057	0.716	0.187
Fasting Triglyceride (mmol/l)	0.917±0.263 (0.57 – 1.50)	0.800±0.365 (0.43 – 2.51) **(n = 36)**	0.533	0.000	0.982	0.038

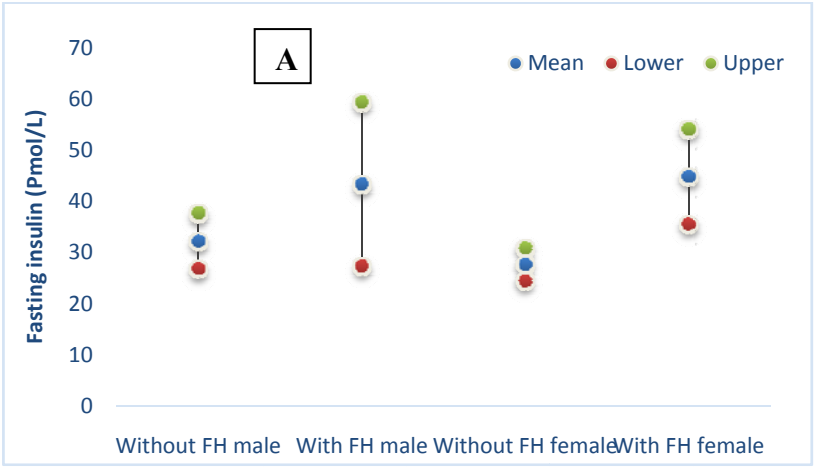
With FH (n = 33)						
B **Variables**	**Male Mean ±SD (Range) (n = 11)**	**Male Mean ±SD (Range) (n = 22)**	**After log10 transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Male P	**Female P**		
Fasting Insulin (pmol/L)	43.23±23.82 (20.88 – 96.30)	44.69±20.96 (12.24 – 97.56)	0.135	0.472	0.589	0.881
Fasting Triglyceride (mmol/l)	0.955±0.386 (0.47 - 1.57)	0.949±0.408 (0.42 – 2.15)	0.458	0.988	0.624	0.958

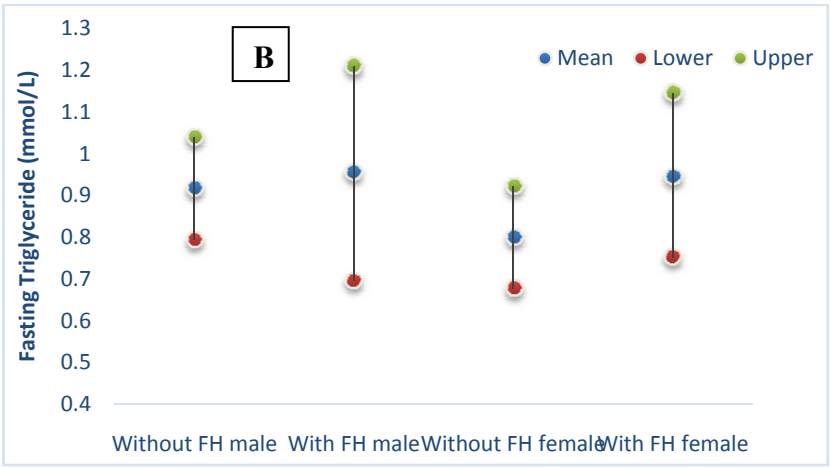
Table 6.6. Influence of family history of type 2 diabetes (FH) on fasting insulin and fasting triacylglycerols in male (n = 31) (A) and in female (n = 59) (B) groups. As groups were positively skewed, log_{10} transformation was done. If the sample had Gaussian distribution and when there was equality of variance, parametric tests were done. Otherwise, non parametric Mann Whitney U test was done.

Male (n = 31)						
A **Variables**	**Without FH Mean ±SD (Range) (n = 20)**	**With FH Mean ±SD (Range) (n = 11)**	**After log10 transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Without FH P	**With FH P**		
Fasting Insulin (pmol/L)	32.11±11.59 (10.02 – 54.48)	43.23±23.82 (20.88 – 96.30)	0.187	0.135	0.896	0.116
Fasting Triglyceride (mmol/l)	0.917±0.263 (0.57 – 1.50)	0.955±0.386 (0.47 -1.57)	0.533	0.458	0.067	0.978

Female (n = 59)						
B **Variables**	**Without FH Mean ±SD (Range) (n = 37)**	**With FH Mean ±SD (Range) (n = 22)**	**After log10 transformation**			
			Shapiro Wilk test		**Levene's test P**	**Student t test (Mann Whitney U test) P**
			Without FH P	**With FH P**		
Fasting Insulin (pmol/L)	27.51±9.59 (8.46 – 50.10)	44.69±20.96 (12.24 – 97.56)	0.057	0.472	0.307	<0.001
Fasting Triglyceride (mmol/l)	0.800±0.365 (0.43 – 2.51) **(n = 36)**	0.945±0.408 (0.42 – 2.15) **(n = 19)**	<0.001	0.988	0.228	(0.063)

Fig.6.1. Influence of family history of type 2 diabetes on male and female samples by comparison of 95% confidence intervals of mean fasting insulin (A) and triglycerides (B) .





Chapter 7

Familiality of type 2 diabetes mellitus upto grandparents are required to partition fasting insulin into groups and subgroups in young non diabetic adults

7.1. Abstract

Hyperinsulinemia is an early change seen before the onset of type 2 diabetes, even before environmental influences build up, generally after 25 years of age. As influences of growth phase are over after 18 years of age, participants in the study were between 18 and 25 years of age. This observational cross sectional study evaluated the influence of familiality of type 2 diabetes mellitus (FH) upto grandparents on fasting insulin in the participants. Clinical and biochemical evaluation of participants were done before inclusion in the study. The cut off levels of levels of various parameters were kept higher to include the influences of insulin resistance. Participants were partitioned into familiality groups I (no FH), II (FH in grandparents, not in parents) and III (FH in parents), and sub partitioned into subgroup IIIa (one parent diabetic) and IIIb (both parents diabetic). Mean fasting insulin increased from group I to II, from II to III and from subgroup IIIa to IIIb. But subgroups of group II, partitioned according to the number of grandparents with diabetes, had similar fasting insulin levels. This study showed that it is possible to establish the baseline fasting insulin reference intervals in each familiality group.

7.2. Introduction

Hyperinsulinemia, decreasing insulin secretion/reserve and metabolic changes are early clinically identifiable end result of multiple genetic and environmental influences that lead to impaired fasting glucose, impaired glucose tolerance and to type 2 diabetes mellitus (DM2) several years later (Weyer et al, 1999; Khan, 2003; Gerich, 2003). Of these three early changes, the objective of this study was to analyse the influence of familiality or family history of type 2 diabetes (FH) on fasting insulinemia for partitioning it into groups, with a long term clinical objectives in the prevention and management of diabetes.

Genetic or familial influences on inuslinemia may be better characterised and partitioned in the young before serious environmental influences build up after the growth phase. The growth phase was considered to be upto eighteen years of age. Serious environmental influences on insulinemia may be restricted before

twenty five years of age. Therefore, the age group for study of the baseline characteristics of fasting insulin was identified to be between 18 - 25 years. There is 'robust' insulin secretion in the young (Brown, 2003; Robertson, 2004; Prentki &Nolan, 2006). Minor insulin secretory deficiency may not influence the low fasting insulin levels and the steady state fasting glucose homeostasis. During the period of progress from hyperinsulinemia to impaired fasting glucose, then to DM2 and finally to advanced diabetes, there is increasing secretory deficiency of insulin (Kahnet al, 1990; Chen et al, 1987; Chen et al,1985; Prentki & Nolan, 2006), resulting in a negative bias of fasting insulin, confounding hyperinsulinemia associated with FH (Haffner et al, 1988). Therefore, young individuals are better suited for study of the influence of FH on fasting insulin. Morning fasting insulin between 7.30 and 8.30 in young individuals without diabetes would have the least diurnal or postprandial variations.

Familiality of diabetes have genetic (McCarthy, 2010; Poulsen et al, 1999) and non genetic components that underlie the early fasting hyperinsulinemia seen in nondiabetic offspring (Haffner et al, 1988), insulin secretory deficiency (Elbein et al, 1999; Stumvoll et al, 2002) and metabolic changes (Tripathy et al, 2003) resulting in aggregation of diabetes in families (Haffner et al.1988; Elbein et al, 1999; Tripathy et al, 2003; Valdez et al, 2007), especially among twins (Poulsen et al, 1999), . There is higher prevalence of diabetes with higher rates of FH (Valdez et al, 2007). The nongenetic inheritance factors may be epigenetic mechanisms or non epigenetic mechanisms such as familial behaviour and culture. The reported increased maternal transmission of diabetes may result from effects of gestational diabetes on fetus leading or from maternally inherited mitochondrial genes (Dabelea et al, 2000; Fetita et al, 2006).

The study problem stated in the title has clinical significance for the development of baseline, partitioned reference intervals and cut off levels of fasting insulin that are influenced by characteristics of FH (Horowitz, 2012; Vague & Nguyen, 2001).

7.3. Aim of Study in this Section:

Evaluation of the influence of family history of type 2 diabetes mellitus upto grandparents on fasting insulin in young adults aged 18 to 25 years.

7.4. Materials and Methods

7.4.1. Case control and sample collection

Healthy cross section of participants (n = 136) from rural population of Kerala state, South India between 18 and 25 years, took part in this observational study. Samples for this study were collected from the year 2010 onwards.

The study was approved by the Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011). Informed written consent was obtained from each participant aged between 18 and 25 years.

7.4.2. Selection of participants, Inclusion and Exclusion criteria

Please refer 2.2 Selection of participants, Inclusion and Exclusion criteriain chapter 2.

7.4.3.Sample collection

Please refer2.3Sample collection in chapter 2.

7.4.4. Analytical control and insulin measurements

Please refer 2.8 Detailed Description of an Immunochemistry Assay, eg. Insulin Assay in chapter 2.

7.4.5. Diagnostic criteria

Please refer 2.18 Diagnostic criteriainchapter 2

7.4.6. Statistical Analysis

Please refer 2.19 Statistical Analysis inchapter 2

7.4.7. Grouping of familiality of Type 2 Diabetes Mellitus

Exposure of FH upto grandparents in the participant were used to partition the sample population into groups I (without FH upto grandparents), II (with FH in grandparents, not in parents) and III (with FH in parents). Groups II and III were sub grouped according to the number of grandparents (1 to 4 as IIa, IIb, IIc, IId) and number parents (1 to 2 as IIIa, IIIb) who have DM2, respectively. Participants of group IIIa was subgrouped according to the maternal [IIIa(M)] and paternal

[IIIa(P)] FH. These were also the expected outcome of the study. Group I was repeatedly analysed for errors. Sample size being small, errors would have marked effect on fasting insulin levels. Few errors resulting in higher levels of fasting insulin, from negative information bias on FH were identified and the samples were shifted to group II.

To start with, sample size was calculated from published literature. It was possible to partition fasting insulin into groups or subgroups only after controlling variances, errors and confounders by repeated revision of data collection. Therefore, we could not systematically document the number of samples excluded at each stage or calculate sample size.

7.5. Results

Characteristics of diabetes-related parameters in the total sample are given (Table 7.1). When the participants without (I) and with (II+III) FH were compared, the maximum difference was seen in fasting insulin ($P < 0.001$) followed by waist circumference ($P = 0.023$) and BMI ($P = 0.069$). Though total triglyceride and LDL cholesterol were also expected to be increased in group with FH due to increased insulin resistance (Kissebah et al, 1976; Sparks & Sparks, 1994;Pivovarova et al, 2013), this was not observed in our finding.

There was a gender bias, female participants ($n = 88$) being more than males ($n = 48$). But it did not affect the outcome of fasting insulin in this study. These issues are discussed in the associated report on gender differences.

The components of HOMA-IR, fasting insulin (μU/ml) and fasting glucose (mmol/l), have numerical weightage that are similar. HOMA-IR and fasting insulin showed very high correlation ($r = 0.977$) indicating that there was little influence of glucose (Fig. 7.1). These results indicated that fasting insulin resistance, represented by HOMA-IR, and fasting insulin were similar and closely related in the sample chosen for the study.

Mean, median and mode were closer together after $\log_{10}$ transformation. One way ANOVA of the $\log_{10}$ transformed values showed that the three familiality groups were different from each other (Table 7.2). Post hoc pair comparison and

95% CI of mean showed that all the three pairs of familiality groups compared were different from each other. Mean fasting insulin increased from group I to II and from II to III (Fig. 7.2). The two methods showed that fasting insulin increased from groups I to II and further increased in III.

Groups II and III were divided into subgroups according to the number of grandparents and parents who were diabetic, respectively. Group IId had no members, as at least one parent was diabetic when all four grandparents had DM2. Fasting insulin in the subgroups IIa, IIb and IIc were not different from each other (P = 0.808). But fasting insulin in each of these subgroups were different from group I (P from <0.001 to 0.001) (Table 7.3; Fig. 7.3). Fasting insulin increased from group IIIa to IIIb (P = 0.015) (Table 7.4). There was no difference between the subgroups of IIIa which was subdivided into IIIa(P) (with paternal FH) and IIIb(M) (maternal FH) (Table 7.4).

7.6. Discussion

Of the earliest three clinically detectable parameters before DM2, hyperinsulinemia, metabolic changes and β-cell dysfunction (Khan, 2003; Gerich, 2003), the first measured as fasting insulin was analysed in this study. Fasting insulin was found to be more reproducible than post glucose load OGTT insulin during the preliminary studies. Fasting glucose and insulin are in a steady state influenced by insulin sensitivity, insulin uptake by tissues, insulin secretion and release of glucose by liver. To summarise the results, FH upto grandparents were found to influence fasting insulin levels in young offspring, which increased from groups I to II, and from II to III. Fasting insulin was not different in the subgroups of II, but increased from subgroup IIIa to IIIb. These criteria were used to partition fasting insulin in the young participants (Table 7.2.).

The risk characteristics of FH on fasting insulin in a proband of this study were presence or absence of DM2 in grandparents or parents; In group III, the number of parents who are diabetic; In group II, irrespective of the number of grandparents who are diabetic, the risk of hyperinsulinemia from fasting insulin levels remain similar and higher than that in group I.

Steps taken to increase internal validity of results (decrease in imprecision, bias and confounders) of fasting insulin for its partitioning were the following: 1. Participants between 18 and 25 years; 2. without any form of diabetes; 3. rural sample population, excluding high income groups as far as possible; 4. control of sample collection.

The decrease in hepatic insulin clearance of plasma insulin is considered as a cause of hyperinsulinemia (Pivovarova et al, 2013; Reaven, 1988; Mittelman et al, 2000). Hyperinsulinemia may be considered as a compensation for insulin resistance which also increases insulin secretion. Hyperinsulinemai is strongly associated with metabolic syndrome (Khan, 2003; Prentki & Nolan, 2006; Pivovarova et al, 2013; Ferrannini & Balkau, 2002).

Table 7.1. Characteristics of diabetes-related parameters and their comparison in participants of groups without (I) and with (II+III) FH. As the variables in (II+III) were not normally distributed, $\log_{10}$ transformation was done. When they were normally distributed and there was equality of variance, Student t test was done; but when they were not, non parametric Mann-Whitney U test was done (in brackets).

Variables	Total sample Mean ±SD Range (n = 136 except for lipid profile n = 132)	Comparison of Groups without (I) and with (II+III) FH					
		I Mean ±SD Range (n = 23)	(II+III) Mean ±SD Range (n = 113)	After $\log_{10}$ transformation			
				Shapiro-Wilk test for normality P		Levene's test for equality of variances P	Student t test (or Mann-Whitney U test) P
				I	(II+III)		
Age (years)	21.87±1.99 18 - 25	21.52±1.73 18 - 25	21.94±2.04 18 – 25	0.659	<0.001	0.540	(0.252)
BMI (kg/m^2)	21.33±3.32 13.03 - 29.1	20.13±2.69 16.22 - 26.12	21.56±3.38 13.03 – 29.1	0.413	0.162	0.231	0.069
Waist circum-ference(cm)	76.54±8.75 57.5 - 104	72.78±8.59 59 - 92.5	77.21±8.65 57.5 – 104	0.529	0.856	0.824	0.023
Fasting Glucose (mmol/L)	5.05±0.54 3.97 - 6.87	4.90±0.53 3.97 - 6.04	5.08±0.54 4.05-6.87	0.352	0.393	0.619	0.153
2 hour OGTT Glucose (mmol/L)	5.48±1.21 2.49 - 9.77	5.23± 0.97 3.77 - 7.28	5.54±1.24 2.5 - 9.77	0.328	0.032	0.442	(0.361)
Fasting Insulin (pmol/L)	36.52±17.82 8.46 - 106.14	22.004±7.43 8.46 - 37.32	39.47±17.89 12.24 - 106.14	0.320	0.057	0.740	<0.001
Total Cholesterol (mmol/L)	4.52 ±1.14 2.85 -7.87 (n = 132)	4.47±0.7 3.42- 6.19	4.70±0.86 2.85 – 7.87 (n = 109)	0.620	0.304	0.638	0.237
Triglycerides (mmol/L)	0.901±0.47 0.42 – 3.34 (n = 132)	0.82±0.226 0.52 – 1.36	0.95±0.48 0.42-3.34 (n=109)	0.402	0.002	0.085	(0.393)
LDL Cholesterol (mmol/L)	2.82±0.901 1.4 – 6.03 (n = 132)	2.68±0.62 1.48 – 4.2	2.95±0.79 1.4 -6.03 (n=109)	0.750	0.848	0.335	0.136
HDL Cholestero (mmol/L)	1.29±0.40 0.65- 2.25 (n = 132)	1.4±0.35 0.88 – 2.2	1.31±0.33 0.65-2.25 (n=109)	0.782	0.433	0.963	0.197

Fig. 7.1. Scatter diagram of fasting insulin and HOMA-IR in the total sample. Log_{10} transformation converted the positively skewed fasting insulin and HOMA-IR to normal distribution for calculation of Pearsons Correlation coefficient (r = 0.977).

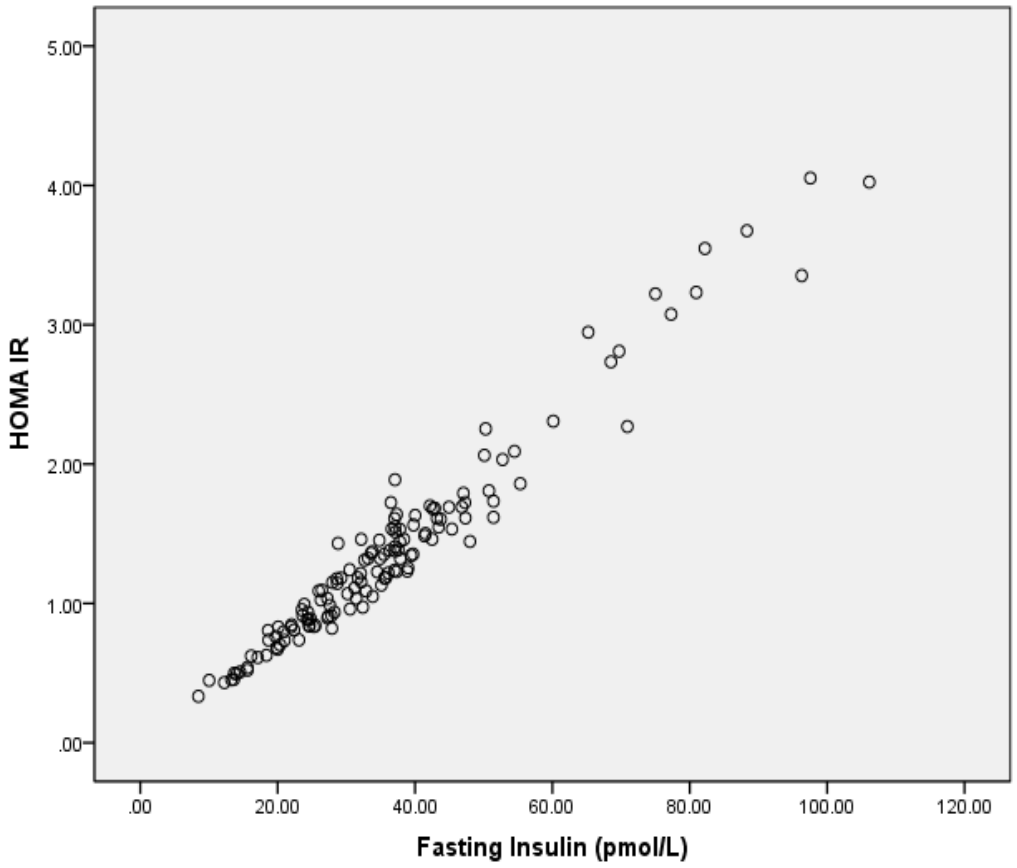


Table 7.2. Comparison of fasting insulin in groups I, II and III. As groups II and III were positively skewed, $\log_{10}$ transformation was done followed by one way ANOVA and post hoc pair comparison in conjunction with Tukey HSD and Bonferroni correction. Fasting insulin in pmol/l before transformation are given in brackets.

Groups		**I** (n = 23)	**II** (n = 60)	**III** (n = 53)
Mean±SD		1.32±0.162 (22.004±7.43)	1.52±0.149 (35.337±12.40))	1.61±0.202 (44.74±21.39)
Median		1.34 (22.02)	1.55 (35.49)	1.59 (38.88)
Mode		1.39 (24.6)	1.57 (37.02)	1.58 (37.8)
95% CI of mean		1.25 - 1.39 (18.79 - 25.22)	1.49 - 1.56 (32.14 - 38.54)	1.55 – 1.66 (38.85 – 50.64)
Shapiro Wilk test, P ($\log_{10}$ transformed)		0.32	0.133	0.346
Test of homogeneity of variances ($\log_{10}$ transformed)		Levene statistic = 2.089 (P = 0.128)		
Range		0.93 – 1.57 (8.46 - 37.32)	1.14 – 1.91 (13.68 - 80.94)	1.09 – 2.03 (12.24 - 106.14)
ANOVA (One way)		F ratio = 22.42 (P = <0.001)		
Post hoc pair comparison (with equal variance)	**Tukey HSD**	I and II, P = <0.001 II and III, P = 0.036 I and III, P = <0.001		
	Bonferroni correction	I and II, P = <0.001 II and III, P = 0.041 I and III, P = <0.001		

Fig. 7.2. Mean plot of fasting insulin in familiality Group I, II and III.

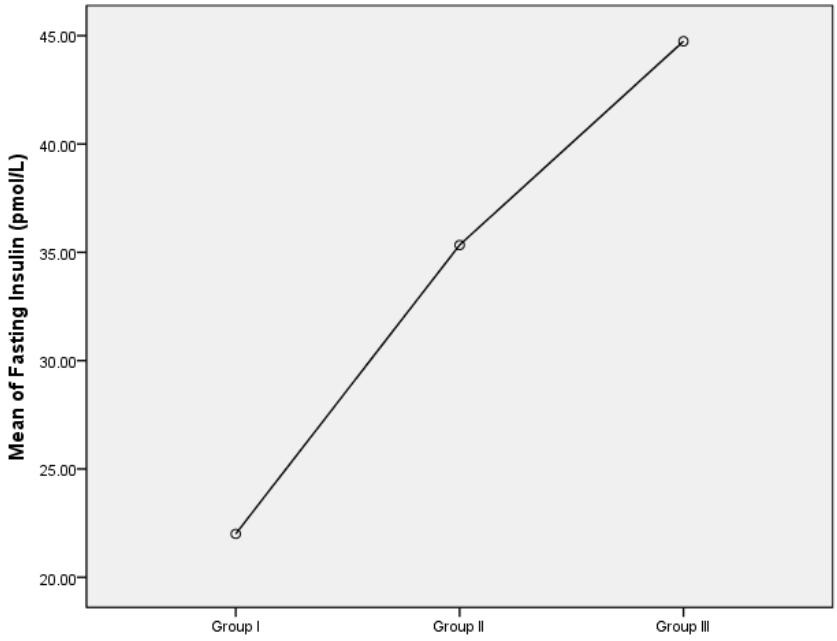


Table 7.3. Comparison of fasting insulin between subgroups IIa, IIb and IIc by nonparametric Kruskal Wallis test, and between each subgroup and group I by Student t test. Fasting insulin in the three subgroups could not be transformed to normal distribution simultaneously. I and IIa were normally distributed. After $\log_{10}$ transformation, I, IIb and IIc were normally distributed. Group IId, n = 0.

Subgroups	**I (n = 23)**	**IIa (n = 23)**	**IIb (n = 29)**	**IIc (n = 8)**
Mean±SD	22.004±7.43	34.26±8.73	35.27±13.71	38.67±16.95
95% CI of mean	18.79 - 25.22	30.48 – 38.03	30.06 - 40.49	24.49 - 52.84
Range	8.46 - 37.32	14.52 - 50.1	13.68 - 80.94	23.88 - 77.28
Shapiro Wilk test, P (before transformation)	0.759	0.144	0.017	0.015
Shapiro Wilk test, P (after $\log_{10}$ transformation)	0.32	-	0.981	0.242
ANOVA (One way) (Kruskal Wallis Test) (groups compared)	-	Test Statistic = 0.426; P = 0.808 (IIa, IIb, IIc)		
Levene's test (groups tested)	-	0.643 (I and IIa)	0.975 (I and IIb)	0.786 (I and IIc)
Student t test between group I and subgroups of II. (groups compared)	-	(before transformation) P = <0.001 (I and IIa)	($\log_{10}$ transformed) P = <0.001 (I and IIb)	($\log_{10}$ transformed) P = 0.001 (I and IIc)

Fig 7.3. Comparison of 95% CI of mean of fasting insulin in Group I with Group IIa, IIb and IIc.

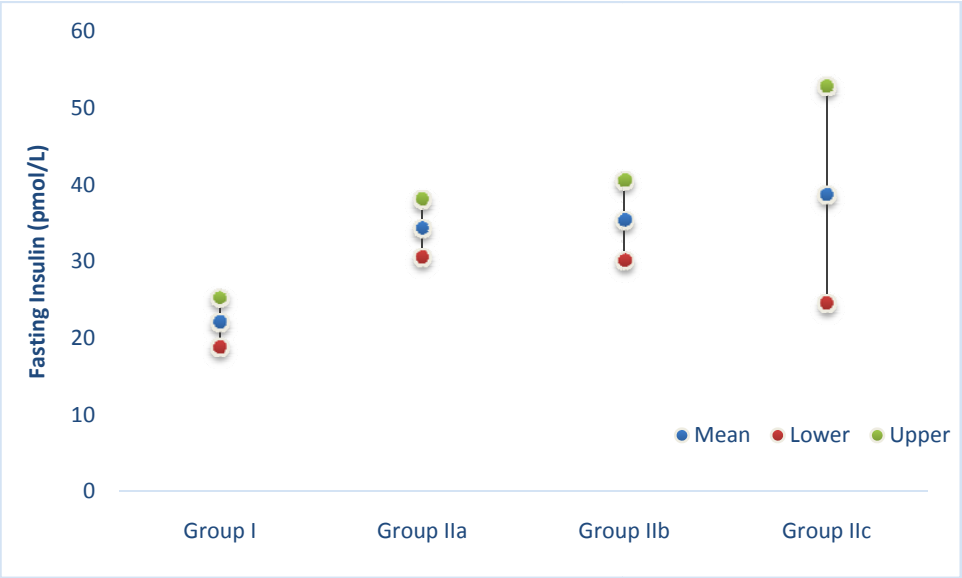


Table 7.4. Comparison of fasting insulin between subgroups IIIa and IIIb, and between [IIIa(M)] and [IIIa(P)]. Data in pmol/l (in brackets) were $\log_{10}$ transformed to convert to normal distribution.

Groups	**IIIa (n = 41)**	**IIIb (n = 12)**	**IIIa(P) (n = 33)**	**IIIa(M) (n = 8)**
Mean ±SD	1.57±0.19 (40.46±17.66)	1.73±0.22 (59.38±26.95)	1.57±0.20 (41.05±19.2)	1.57±0.11 (38.02±9.4)
95% CI of mean	1.51 – 1.63 (34.88 – 46.03)	1.59 – 1.86 (42.26 – 76.50)	1.5 – 1.64 (34.24 – 47.86)	1.48 – 1.66 (30.16 – 45.88)
Shapiro Wilk test, P (after $\log_{10}$ transformation)	0.259	0.322	0.477	0.35
Levene's test (groups tested)	0.228 (IIIa and IIIb)		0.263 [IIIa(P) and IIIa(M)]	
Student t test (groups compared)	P = 0.015(IIIa and IIIb)		P = 0.973 (IIIa (P) and IIIa (M))	

Chapter 8

Gender differences in fasting insulin concentrations and in its distribution in young adult participants

8.1. Abstract

Influence of gender differences have been reported in the prevalence of type 2 diabetes (DM2) in offspring. But the gender differences in fasting insulin before or after partitioning young offspring according to familiality of DM2 (FH) have not been reported, and was analysed in this observational cross sectional study. Fasting insulin was assayed in young individuals partitioned into males and females and sub partitioned into groups I (without FH upto grandparents), II (with FH in grandparents, not in parents), and III (with FH in parents). One way ANOVA of fasting insulin showed that the three groups were different in males (P = 0.001) and females (P = <0.001), and males had consistently higher mean insulin than females in groups II (P = 0.051) and III (P = 0.413). Fasting insulin in females from groups II and III were normally distributed, but not in males. The influence of gender on fasting insulin and its distribution are required for understanding future risk of hyperinsulinemia.

8.2. Introduction

Multiple genetic/familial and environmental factors contribute to the aetiology of type 2 diabetes (Chen et al, 1985; Doria et al, 2008). The genetic factors have lead to clustering of type 2 diabetes in families (Gottlieb, 1980; Li et al, 2000; Hilding 2006), its heritability (Hilding et al, 2006; Almgren et al, 2011) and the high degree of concordance of diabetes in monozygotic twins (Poulsen et al, 1999: Kyvik et al, 1995). There are racial and ethnic differences in the prevalence, insulin sensitivity, insulin response and risk of diabetes. There are also differences in the prevalence of diabetes and impaired glucose tolerance according to maternal or paternal history of diabetes (Kodama et al, 2013; Mitchell et al, 1993; Valdez et al, 2007). There is correlation between prevalence of type 2 diabetes and genetic admixture in hybrid populations (Serjeantson et al, 1983; Brosseau et al, 1979).

Increase in the prevalence of type 2 diabetes (DM2), impaired glucose tolerance and hyperinsulinemia (Gottlieb, 1980; Li et al, 2000; Mitchell et al, 1993; Valdez et al, 2007). In offspring of parents with DM2 has been reported. Gender

differences in the prevalence of DM2 in the offspring, related to paternal and maternal familiality or family history of DM2 (FH) have also been reported (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000). But what was not reported and the objective of this study are the gender differences in familiality and in fasting insulin of offspring, as follows: Partitioning of a young adult proband or participant of this study according to their gender and FH upto grandparents; Prevalence of DM2 and its gender differences in parents and grandparents of the partitioned participants; Gender difference in fasting insulin in these partitioned participants. These are required for future estimation of the risk of hyperinsulinemia and DM2 in the proband.

Gender differences in the prevalence of DM2 with paternal and maternal familiality of DM2 in Mexican Americans and non Hispanic whites from the San Antonio Heart Study and in participants who are primarily Caucasians in the Framingham Offspring Study had been reported earlier (Mitchell et al, 1993; Stern et al, 1984; Meigs et al, 2000). The four major findings in the report by Mitchell et al (Mitchell et al, 1993) were: Men with parental history of DM2 had higher prevalence of diabetes and impaired glucose tolerance than men without parental history; In men, the prevalence was equally high regardless of which parent or both parents; Women with only a maternal history of DM2 was associated with higher prevalence of DM2 and impaired glucose tolerance; In women, there was no difference in the prevalence of DM2 between women with a paternal history of diabetes and women with no parental history of diabetes. The Framingham Offspring Study showed that offspring with maternal diabetes and an age of onset of less than 50 years had increased risk of diabetes (Meigs et al, 2000).

The prevalence of diabetes is higher among the offspring of diabetic mothers than among the offspring of non diabetic mothers or diabetic fathers. These observations have been made in clinical and experimental studies (Kasperska-Czyzyk et al,1996; Pettitt et al, 1988; Thomas et al, 1994; Han et al, 2007). Exposure in utero to a diabetic mother is associated with a higher risk of obesity, IGT and DM2 in offspring (Pettitt et al, 1988; Dabelea et al, 2000; Silverman et al, 1995). Insulin secretion has been shown to be abnormal in offspring of diabetic mothers (Aerts et al,1988).

Familiality of DM2 may be due to genetic and non genetic factors (Han et al, 2007; Dabelea et al, 2000; Doria et al, 2008). Non genetic factors may be epigenetic factors or non epigenetic issues such as cultural, behavioural and environmental aspects specific to the family. Familiality may also result from intrauterine influences on fetal growth and development, which can lead to changes later in life (Dabelea et al, 2000; Silverman et al, 1995; Aerts et al,1988). The intrauterine effects and mitochondrial inheritance may also contribute to maternal transmission of diabetes.

Prevalence of diabetes in India showed an increase, as reported from 1988 onwards and was predicted to increase further (Ramachandran et al, 1988; Wild et al, 2004; Mohan et al, 2007; Sadikotet al, 2004; Whiting et al, 2011). In the year 2000, India had the highest number of individuals with diabetes, and was predicted to increase fastest upto 2030 (Wild et al, 2004; Whiting et al, 2011). Prevalence was higher in urban when compared to rural population (Ramachandran et al,1988; Mohan et al, 2007; Sadikotet al, 2004; Vijayakumar et al, 2009; Ramachandran et al, 1992; Ramachandran et al, 2001). High prevalence of diabetes was reported in Kerala state, from where this report originates (Vijayakumar et al, 2009). There was increase in the prevalence of diabetes with age upto 60 - 69 years in urban population. Thereafter, the prevalence decreased with increase in age above 69 years. This was seen when the prevalence was 8.6% to 19.5% in different states of India from 2001 to 2006 (Mohan et al, 2007). In another report from Australia, with a prevalence of 4.4% (Collins & Kalisch, 2011), the prevalence increased upto 55 – 59 years of age and thereafter decreased. With increase in prevalence of diabetes, there is a progressive decrease in the average age of onset of diabetes in India (Mohan et al, 2007). A report from South India on familial aggregation of DM2 showed that there was absence of excess maternal transmission.

8.3. Aim of Study in this Section

Influences of gender and family history of type 2 diabetes mellitus young participants before and after partitioning the sample according to gender and family history of type 2 diabetes mellitus.

8.4.Materials and Methods

8.4.1. Study Settings and Design

Healthy cross section of participants (n = 136) between 18 and 25 years, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala from rural population of Central Kerala.

The Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011) approved the study. Informed written consent was obtained from each participant aged between 18 and 25 years.

8.4.2. Selection of participants, Inclusion and Exclusion criteria

Please refer 2.2 Selection for participants, and for Inclusion and Exclusion criteriain Chapter 2.

8.4.3.Sample collection

Please refer2.3. forsample collection in Chapter 2.

8.4.4. Analytical control and insulin measurements

Please refer 2.8 for detailed description of an Immunochemistry Assay, eg. Insulin Assay in Chapter 2.

8.4.5. Diagnostic criteria

Please refer 2.18 for diagnostic criteriainChapter 2

8.4.6. Statistical Analysis

Please refer 2.19 for Statistical Analysis inChapter 2

8.5. Results

Characteristics of some of the Insulin and DM2-related parameters were evaluated and their gender differences were compared in groups I (Table 8.1) and (II+III) (Table 8.2). Mean of all variables were in the healthy reference range. As

expected, HDL cholesterol was higher in females of both groups (P = 0.002; P < 0.001). There was no gender difference in fasting insulin from group I (P = 0.826). In group (II+III) mean fasting insulin was higher in males but the increase was not statistically significant (P = 0.149). But fasting triglycerides, which is related to insulin resistance, was elevated in males with FH (P = 0.001) but was not elevated in males without FH (P = 0.805). Though BMI and fasting glucose were higher in males of group I, the differences appear to be less related to insulin resistance as the means were close to each other within the reference interval and may be due to increased body size of males. There was no gender difference in any other parameter. As the samples were partitioned according to gender, the gender bias with higher females (n = 88) than males (n = 48) should not affect the outcome of this study.

Familiality of type 2 diabetes influences fasting insulin in young males and in young females

Total sample was divided into males and females, and then they were subdivided into Groups I, II and III. The $\log_{10}$ transformed fasting insulin in groups was normally distributed and had equality or homogeneity of variances. One way ANOVA showed difference between the groups in males (P = 0.001) (Table 8.3) and also in females (P = < 0.001) (Table 8.4). Post hoc pair comparison, after corrections for unequal sample sizes and multiple comparisons, showed that there was difference between groups I and II, and I and III in both male and female samples. But there were no (males) or borderline (females) difference observed between groups II and III (Table 8.3 and Table 8.4).

There was no gender difference in mean fasting insulin levels from group I (Fig. 8.1; Table 8.5). But in groups II and III there was a consistent increase of mean fasting insulin from males as compared to that from females (Fig. 8.1). The increase was statistically significant in group II but not in groups I and III as seen with Student t test (Table 8.5). Visual comparison of the 95% CI of means also indicated similar observations (Fig. 8.2). These observations indicated that there was relatively higher fasting insulin in males with FH but not in males without FH.

The distribution of fasting insulin was also analysed in groups II and III (Table 8.6). There were more outliers in the male fasting insulin sample showing

that male sample was more positively skewed. Tests of normality also showed that in groups II and III, male samples of fasting insulin did not have Gaussian distribution, they were positively skewed.

8.6. Discussion

Premenopausal women with regular menstrual cycles have enhanced insulin sensitivity when compared to adult men (Jarvis et al, 2013; Park et al, 2003; Yki-Jarvinen 1984) and was proposed to be the cause of reduced incidence of DM2 in premenopausal women (Park et al, 2003; Yki-Jarvinen 1984). After menopause, there is increase in body fat mass, circulating triglycerides, LDL cholesterol, inflammatory markers and free fatty acids (Carr, 2003; Pfeilschifter et al, 2002; Sites et al, 2002). These changes seen after menopause coincided with the sudden decreased insulin sensitivity. Similar observations were made after ovariectomy (Jarvis et al, 2013; Yki-Jarvinen 1984; Carr, 2003). Mice with global estrogen receptor deficiency developed hepatic insulin resistance and there was decreased insulin suppression of hepatic glucose production during a euglycemic, hyperinsulinemic clamp (Bryzgalova et al,2006). However, these mice did not show increased insulin resistance in skeletal muscles. The higher levels of estrogen during pregnancy may increase insulin resistance (Jarvis et al, 2013) which may explain gestational diabetes in women who are predisposed to DM2.

Gender differences in fasting insulin is due to the relative hyperinsulinemia in young males with FH

The difference in the distribution of fasting insulin was related to increased positive skewing and increase in proportion of positive outliers in males. These gender differences were related to increased fasting insulin in males of group II and III, as compared to females (Table 8.5 and Fig. 1 and 2). There was no gender difference in mean fasting insulin in group I. The increased insulin resistance in males with FH may be further supported by the increased fasting triglycerides in males of group (II+III) (Table 8.2). Hyperinsulinemia and increased triglycerides were related to insulin resistance (Laws and Reaven, 1992; Fan et al, 2011).

Significance of groups I, II and III, and proposed partitioning of fasting insulin in young participants (Chapters 7 and 8; Table 8.7)

The proposed partitioning of fasting insulin is given in Table 8.7. The justification for the partitioning is as follows. In group I, there was no gender difference in fasting insulin and triglycerides (Table 8.5 and Fig. 8.1 and 8.2). Group I was without hyperinsulinemia, thus giving the group the lowest baseline reference interval character of fasting insulin to be estimated later.

In group II, fasting insulin was increased, there was gender difference but could not be subpartitioned (Chapter 7). There was no difference in the concentration of fasting insulin in the subgroups of group II (Chapter 7). For the same reason, there were no members in subgroup IId. Members of group II may have relatively higher age of onset of DM2.

In group III, the number of parents with diabetes (IIIa and IIIb) increased fasting insulin in the participant (Chapter 7). Group III indicates lower age of onset of DM2. Fasting insulin was increased in males. We expected mean fasting insulin in group III males and females to be much higher than that in group II, but it was not so (Table 8.3 and 8.4). The possible argument for the phenomenon is the quantitative secretory deficit of insulin confounding hyperinsulinemia of group III, and the difficulty in evaluation of this in clinical setting. Same explanation may also hold for the lower gender difference of fasting insulin group III.

Against our expectations, there was no significant difference between groups II(m) and III(m), and there was only borderline difference between II(f) and III(f) (Table 8.3 and 8.4). But there was difference between groups II and III before subpartitioning into males and females (Chapter 7). The explanations are the increased secretory deficiency of insulin in group III(m) and III(f) confounding hyperinsulinemia.

There is uncertainty about the relationship between insulin resistance and hyperinsulinemia, nor the relationship between familiality of DM2 and hyperinsulinemia. It was proposed that hyperinsulinemia may be a compensation for insulin resistance that leads to increased insulin secretion and decreased hepatic clearance of insulin (Pivovarova et al, 2013; Mittelman et al, 2000; Ferrannini and Balkau, 2002).

Table 8.1. Comparison of diabetes related variables in male and female participants from group I. All variables in group I males and females were normally distributed and were of equal variance, except HDL cholesterol. Student t test was done when they were normally distributed and were of equal variance; when they were not, non parametric Mann Whitney U test was done (given in brackets). For all variables n = 136, except lipid profile n = 132.

Variables	Group I (male and female)					
	Male Mean ±SD (Range) (n = 7)	Female Mean ±SD (Range) (n = 16)	Shapiro Wilk		Levene's test P	Student t test (Mann Whitney U test) P
			Male P	Female P		
Age (years)	21.14±2.47 (18 - 25)	21.69±1.35 (19 - 24)	0.255	0.506	0.073	0.500
BMI (kg/m^2)	22.00±2.51 (19.16 - 26.12)	19.32±2.4 (16.22 - 24.89)	0.155	0.108	0.897	0.024
Waist circum-ference (cm)	76.21±9.59 (65.0 – 92.5)	71.28±7.97 (59 - 92)	0.641	0.236	0.562	0.212
Fasting Glucose (mmol/L)	5.25 ±0.62 (4.44 - 6.04)	4.75±0.41 (3.97 – 5.5)	0.498	0.437	0.085	0.032
2 hour OGTT Glucose (mmol/L)	5.34±1.05 (3.77 - 6.48)	5.18±0.96 (3.84 - 7.28)	0.514	0.461	0.593	0.723
Fasting Insulin (pmol/L)	21.47±6.66 (10.02 - 29.22)	22.24±7.94 (8.46 - 37.32)	0.722	0.639	0.797	0.826
Total cholesterol (mmol/L)	4.32±0.68 (3.5 – 5.26)	4.53 ±0.72 (3.42 – 6.19)	0.428	0.707	0.899	0.519
Trigly-cerides (mmol/L)	0.801±0.26 (0.52 – 1.25)	0.83±0.22 (0.59 - 1.36)	0.544	0.079	0.583	0.805
LDL Cholesterol (mmol/L)	2.86±0.62 (2.15 – 3.81)	2.61±0.63 (1.48 – 4.2)	0.277	0.511	0.809	0.374
HDL Cholesterol (mmol/L)	1.1±0.15 (0.88 – 1.3)	1.55±0.32 (1.06 – 2.2)	0.707	0.702	0.027	(0.002)

Table 8.2. Comparison of diabetes related variables in male and female participants from Group (II + III). Log_{10} transformation converted some variables in group (II+III) to normal distribution and with equality variance. Student t test was done when they were normally distributed and were of equal variance; when they were not, non parametric Mann Whitney U test was done (given in brackets). For all variables n = 136, except lipid profile n = 132.

Variables	Group (II+III) (male and female)					
	Male Mean ±SD (Range) (n = 41)	Female Mean ±SD (Range) (n = 72)	After log_{10} transformation			
			Shapiro Wilk		Levene's test in males and females P	Student t test (Mann Whitney U test) P
			Male P	Female P		
Age (years)	21.41±2.53 (18 - 25)	22.24±1.65 (18 - 25)	0.001	0.001	<0.001	(0.096)
BMI (kg/m^2)	22.19±3.7 (13.03 - 29.1)	21.2±3.15 (15.47-28.08)	0.141	0.087	0.302	0.180
Waist circum-ference (cm)	77.86±10.75 (57.5 - 104)	76.84±7.26 (60 - 95)	0.746	0.595	0.035	(0.783)
Fasting Glucose (mmol/L)	5.15 ±0.45 (4.42 - 6.87)	5.04 ±0.58 (4.05 - 6.71)	0.062	0.157	0.002	(0.256)
2 hour OGTT Glucose (mmol/L)	5.60 ±1.29 (2.5 - 9.77)	5.51 ±1.22 (2.77 - 9.46)	0.155	0.334	0.647	0.716
Fasting Insulin (pmol/L)	42.96±21.21 (19.86-106.14)	37.49 ±15.5 (12.24-97.56)	0.051	0.261	0.861	0.149
Total cholesterol (mmol/L)	4.81±1.1 (2.85 – 7.87)	4.64±0.67 (3.63 – 6.37) (n = 68)	0.820	0.091	0.006	(0.745)
Trigly-cerides (mmol/L)	1.11±0.56 (0.47 – 3.34)	0.85±0.40 (0.42 – 2.51) (n = 68)	0.165	0.002	0.577	(0.001)
LDL Cholesterol (mmol/L)	3.14±1.01 (1.66 – 6.03)	2.84±0.59 (1.4 – 4.3) (n = 68)	0.515	0.346	0.022	(0.342)
HDL Cholesterol (mmol/L)	1.16±0.26 (0.65 – 2.25)	1.40±0.34 (0.8 – 2.18) (n = 68)	0.023	0.395	0.058	(<0.001)

Table 8.3. Comparison of fasting insulin between familiality groups I, II and III in males (n = 48) by one way ANOVA and post hoc pair comparison. Log_{10} transformation converted all male groups to normal distribution and Levene's test showed homogeneity of variances. Mean and its Standard deviation after log_{10} transformation are given in brackets.

Groups		**I(m)** **n = 7**	**II(m)** **n = 22**	**III(m)** **n = 19**
Mean±SD, pmol/l		(1.31±0.16) 21.47±6.66	(1.57±0.156) 39.80±15.41	(1.64±0.2) 48.23±25.34
Shapiro Wilk, P		0.241	0.206	0.095
Test of homogeneity of variances		Levene statistic = 0.700, P = 0.502		
One way ANOVA		F = 8.971; P = 0 .001		
Post hoc pair comparison (with equal variance)	**Tukey HSD**	P = 0.003 [I(m) and II(m)] P = 0.486 [II(m) and III(m)] P = <0.001 [I(m) and III(m)]		
	Bonferroni correction	P = 0.004 [I(m) and II(m)] P = 0.762 [II(m) and III(m)] P = <0.001 [I(m) and III(m)]		

Table 8.4. Comparison of fasting insulin between familiality groups I, II and III in females (n = 88) by one way ANOVA and post hoc pair comparison. Log_{10} transformation converted all female groups to normal distribution and Levene's test showed homogeneity of variances. Mean and its Standard deviation after log_{10} transformation are given in brackets.

Groups		**I(f)** **n = 16**	**II(f)** **n = 38**	**III(f)** **n = 34**
Mean±SD, pmol/l		(1.32±0.17) 22.24±7.94	(1.5±0.14) 32.75±9.57	(1.59±0.20) 42.79±18.96
Shapiro Wilk, P		0.604	0.102	0.359
Test of homogeneity of variances		Levene statistic = 1.86, P = 0.161		
One way ANOVA		F = 13.561; P = <0.001		
Post hoc pair comparison (with equal variance)	**Tukey HSD**	P = 0.002 [I(f) and II(f)] P = 0.061 [II(f) and III(f)] P = <0.001 [I(f) and III(f)]		
	Bonferroni correction	P = 0.002 [I(f) and II(f)] P= 0.072 [II(f) and III(f)] P= <0.001 [I(f) and III(f)]		

Fig. 8.1. Comparison of mean fasting insulin in pmol/l from male and female samples of Groups I, II and III.

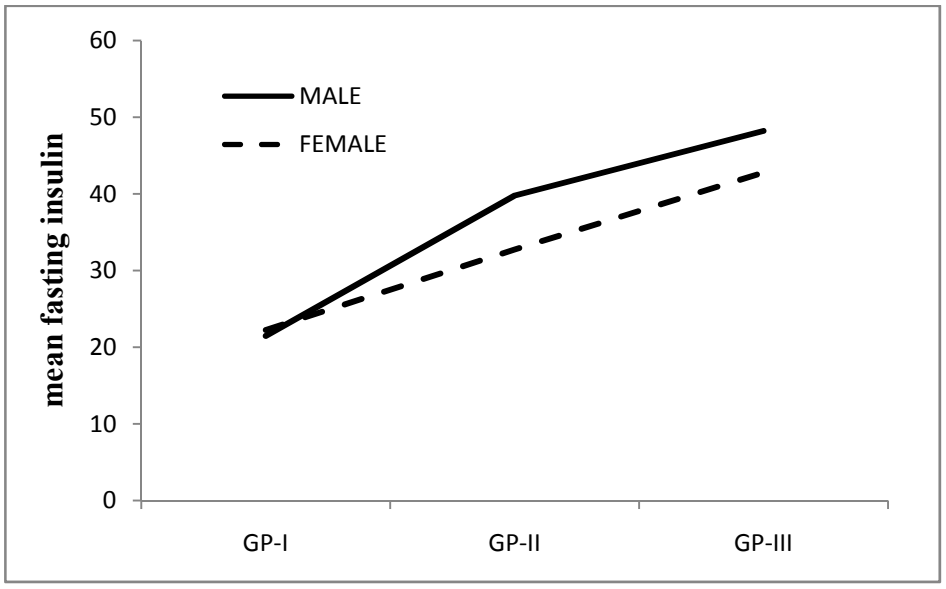


Table 8.5. Comparison of fasting insulin between males (n = 48) and females (n = 88) in each group by Student t test. Log_{10} transformation converted all male and female groups to normal distribution and Levene's test showed homogeneity of variances.

Fasting Insulin in Groups	Male 95% CI of mean (pmol/L)	Female 95% CI of mean (pmol/L)	after log_{10} transformation			
			Shapiro Wilk		Levene's test in males and females P	Student t test (Mann Whitney U test) P
			Male P	Female P		
I	15.32 - 27.63	18.01 - 26.47	0.241	0.604	0.950	0.901
II	32.97 - 46.63	29.61 - 35.89	0.206	0.102	0.965	0.051
III	36.02 - 60.45	36.17 - 49.41	0.095	0.359	0.872	0.413

Fig. 8.2. Visual comparison of 95% CI of mean fasting insulin between males and females in groups I, II and III.

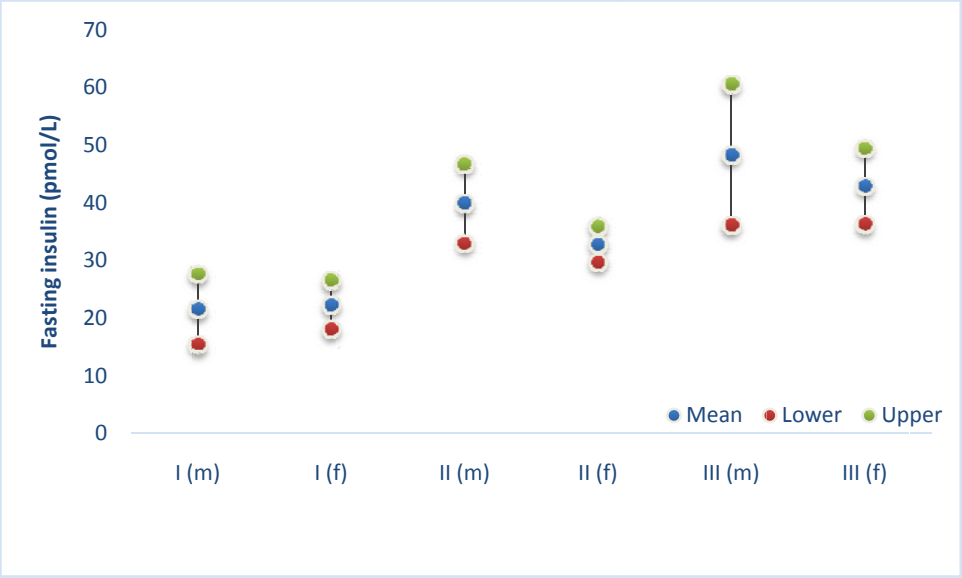


Table8.6. Gender differences in the distribution of fasting insulin in groups II and III sub partitioned according to gender. Proportion of the outliers, statistic of tests of normality and coefficient of skewness are given.

Groups		N	Outliers		Coefficient of skewness	Test of Normality			
						Anderson-Darling		Shapiro Wilk	
			Number	Proportion		statistic	P	statistic	P
I		23	0	0	0.259	0.209	0.843	0.973	0.759
II	Male	22	2	0.091	1.44	1.131	<0.005	0.852	0.004
	Female	38	1	0.026	0.18	0.344	0.47	0.977	0.621
III	Male	19	4	0.211	1.32	1.633	<0.005	0.804	0.001
	Female	34	1	0.029	0.83	0.596	0.113	0.948	0.105

Table 8.7. Proposal for partitioning fasting insulin into groups and subgroups. Fasting insulin levels in subgroups of group II were not different and sub partitioning was not done. There were no gender differences of fasting insulin in group I.

Partitioning the familiality of type 2 diabetes mellitus into Groups and Subgroups	**n (in males and females)**	**Criteria of partitioning familiality of type 2 diabetes**
I	23	Parents and grandparents are not diabetic. No gender difference in fasting insulin.
II **[II(m) + II(f)]**	60 (22 + 38)	Parents are not diabetic but one or more of grandparents are diabetic. Sub partitioned into male and female subgroups.
III **(IIIa + IIIb)** **[III(m) + III(f)]**	53 (41 + 12) (19 + 34)	Any one (IIIa) or both (IIIb) parents are diabetic. Sub partitioned into male (m) and female (f) subgroups.
(II + III) **[(II+III)(m) + (II+III)(f)]**	113 (41 + 72)	Participants with family history of diabetes in parents or grandparents. Sub partitioned into male and female subgroups.
Total Sample **(I+II+III)**	136	All participants in the study.
Noteon Group II: If in a participant, FH in grandparents could not be established and parents do not have diabetes but siblings of parents have diabetes, then the participant was then included in Groups IIa for fasting insulin calculations.		

CHAPTER 9

Gender difference in fasting C peptide in groups with family history of type 2 diabetes mellitus

9.1. Abstract

C peptide represents the secretory components of fasting insulin. Fasting insulin levels are contributed by a number of factors such as FH, male gender, hepatic clearance of insulin, increased insulin secretion and by regulation of insulin degradation. In this study we evaluated the influence of FH and gender on fasting C peptide levels in young adults aged 18 – 25 years. Mean fasting C peptide in group (II+III) was higher than in group I, but with boarder line significance (P = 0.076). One way ANOVA of fasting C peptide in groups I, II and III showed no significant difference between the groups. Even after the partitioning the samples into male and female, group I, II and III were similar. But there was a clear gender difference between male and female samples in group II (P =0.041), in group III (P = 0.031) and in group (II+III) (P = 0.007). Analysis of 95% CI of mean also showed increase of fasting C peptide in males as compared to females in groups II, III, (II+III). These results indicate that FH has no influence on fasting C peptide levels. There was a strong gender difference in group II and III and not in group I for fasting C peptide. These results confirmed that gender difference in fasting C peptide was seen in individuals with FH.

9.2. Introduction

There is a relationship between hyperinsulinemia and insulin resistance (Khan, 2003). There is also a relationship between hyperinsulinemia and family history of type 2 diabetes mellitus (Haffner et al, 1988). But is uncertainty about these relationships. The factors that contribute to hyperinsulinemia are insulin secretion, male gender (Chapter 8), family history of type 2 diabetes (Chapter 7), hepatic insulin clearance and the rate of insulin degradation ((Pivovarova et al, 2013; Mittelman et al, 2000; Ferrannini and Balkau, 2002). Insulin secretion is inversely related to insulin sensitivity or is directly related to insulin resistance. There is a hyperbolic relationship between insulin sensitivity and insulin secretion (Khan, 2003). Insulin secretion may be represented by the level of C peptide. The fasting state is considered as steady state where there is a stable level of circulating insulin, with a steady state glucose release from liver. The release of hepatic glucose is inhibited by circulating insulin. Insulin resistance requires increased

level of insulin, for maintaining hepatic glucose release. Insulin resistance is also compensated by increased insulin secretion and proliferation of β cells of pancreas (Weyer et al, 1999; Khan, 2003; Gerich, 2003).

Family history of type 2 diabetes mellitus influences fasting insulinemia (Haffner et al, 1988). Fasting hyperinsulinemia is contributed by a number of factors, one of which may be insulin secretion. If insulin secretion contributes to hyperinsulinemia, then it is important to verify whether family history of type 2 diabetes mellitus influences insulin secretion. One of the study problems of this section is to evaluate the effects of family history on fasting C peptide.

Insulin resistance is important in the progress towards type 2 diabetes mellitus and in established diabetes. Compared with age matched men, women with normal menstrual history have enhanced insulin sensitivity. This contributes to the reduced incidence of type 2 diabetes mellitus observed in premenopausal women (Park et al, 2003; Yki-Jaryinenh, 1984).

Insulin secretory deficiency also contributes to pathogenesis of type 2 diabetes mellitus (Khan, 2003; Prentki & Nolan, 2006). Premenopausal women with regular menstrual cycles have enhanced insulin sensitivity when compared to adult men (Jarvis et al, 2013; Park et al., 2003; Yki-Jarvinen 1984) and was proposed to be the cause of reduced incidence of DM2 in premenopausal women (Park et al., 2003; Yki-Jarvinen 1984). After menopause, there is increase in body fat mass, circulating triglycerides, LDL cholesterol, inflammatory markers and free fatty acids (Carr, 2003; Pfeilschifter et al, 2002; Sites et al, 2002).

Exposure in utero to a diabetic mother is associated with a higher risk of obesity, IGT and DM2 in offspring (Pettitt et al, 1988; Dabelea et al., 2000; Silverman et al., 1995). Insulin secretion has been shown to be abnormal in offspring of diabetic mothers (Aerts et al.,1988).

9.3. Aim of study in this section

Analysis of the influence of family history of type 2 diabetes mellitus upto grandparents on fasting C peptide and their gender differences.

9.4. Materials and method

9.4.1. Study Settings and Design

Healthy cross section of participants (n = 90) between 18 and 25 years, took part in this observational cross sectional study. Samples for this study were collected from relatives and friends of students, research fellows and laboratory staff of Amala from rural population of Central Kerala.

The Institutional Research and Ethics Committees (AIMSIEC/01/2011 dated 9/3/2011) approved the study. Informed written consent was obtained from each participant aged between 18 and 25 years.

9.4.2. Selection of participants, Inclusion and Exclusion criteria

Please refer section 2.2 Selection of participants, Inclusion and Exclusion criteriain chapter 2.

9.4.3. Sample collection

Please refer2.3Sample collection in chapter 2.

9.4.4.Analytical measurements of C peptide

Please refersection 2.9. Assay of C peptidein chapter 2.

9.4.5. Diagnostic criteria

Please refer 2.18 Diagnostic criteriainchapter 2

9.4.6. Statistical Analysis

Please refer 2.19 Statistical Analysis inchapter 2

9.5. Results

Characteristics of various C peptide and diabetes related parameters in groups without (I) and with (II+III)) family history of type 2 diabetes upto grandparents were evaluated. The mean values were within the reference interval of various parameters and the range was within the cutoff levels in the inclusion / exclusion criteria (Table 9.1). The differences between group I and group (II+III) of these parameters were evaluated (Table 9.1). Maximum increase was seen in fasting insulin ($P < 0.001$). This was followed by LDL cholesterol, total cholesterol and fasting C peptide. Though triglycerides were increased in the group with family history of type 2 diabetes, the increase was not statistically significant.

C peptide represents insulin secretion and insulin secretion contributes to fasting insulin. Therefore, the relationship between fasting insulin and fasting C peptide were analysed by X-Y scatter diagram (Fig 9.1). There was a direct relationship between fasting insulin and fasting C peptide.

The influence of family history of type 2 diabetes on fasting C peptide in groups I, II and III were analysed by one way ANOVA (Table 9.2). There was no significant difference seen between the groups (P = 0.200). Post hoc pair comparison between the three groups also showed no difference between any of the three groups including group I and III. These results showed that there was no influence of family history of type 2 diabetes on C peptide, unlike that seen on fasting insulin.

The above results were further verified after partitioning the sample into male and female (Table 9.3). Family history of type 2 diabetes did not have any influence on fasting C peptide in groups I, II and III of the male sample (P = 0.0172) and on the female sample (P = 0.512).

As there was no influence of family history of type 2 diabetes on fasting C peptide, the influence of gender on fasting C peptide was evaluated. Samples were partitioned into groups I, II and III and each group was sub partitioned into male and female. Fasting C peptide was compared between the male and female samples of groups I, II, III and (II+III) by parametric student t test or by non parametric Mann Whitney U test (Table 9.4). It was observed that there was no gender difference in group I male and group I female. But there was a gender difference between group II male and group II female (P = 0.041). There was also a gender difference between group III male and group III female (P = 0.031) and between group (II+III) male and group (II+III) female (P= 0.007). These results showed that group II and III individually and in combination showed gender difference.

The 95% CI of fasting C peptide was compared between male and female sample of group II, III and (II+III) (Fig. 9.2). It was observed that the male sample had higher mean and 95% CI of mean in the male samples were above but overlapping in group II and III but were clearly different in group (II+III). These results clearly showed that there is gender difference in the groups with family history of type 2 diabetes.

Therefore, the above results indicates that fasting C peptide is not influences by family history of type 2 diabetes but the gender difference was seen in the groups with family history of type 2 diabetes upto grandparents.

9.6. Discussion

Family history influences fasting hyperinsulinemia (Haffner et al, 1988). Fasting hyperinsulinemia is contributed by a number of factors, including insulin secretion. Therefore, the first part of this section analyzed the influence of family history on the fasting C peptide. When participants were partitioned into 3 groups according to family history of type 2 diabetes upto grandparents, it was observed that there was no influence on fasting C peptide levels (Table 9.2). Even after partitioning the total samples into male and females followed by sub partitioning into group I, II and III, it was observed that there was no influence of family history on fasting C peptide (Table 9.3). Therefore, unlike fasting insulin, fasting C peptide was not influenced by family history of type 2 diabetes mellitus. In the male sample there is a moderate increase of fasting C peptide from group I male (mean 0.485) to group II male (mean 0.70). This increase in fasting C peptide was not statistically significant.

But there was a clear gender difference of fasting C peptide in group II and group III (Table 9.4). There is increase in fasting C peptide in the male sample of group II, III and (II+III). But there was no gender difference in group I. These results indicate that gender difference in fasting C peptide existed when there was family history of type 2 diabetes mellitus. Therefore, family history of type 2 diabetes mellitus showed higher fasting C peptide level in males indicating increased insulin secretion. This finding is consistent to the reported observation of reduced incidence of type 2 diabetes mellitus in premenopausal women (Park et al, 2003; Yki-Jaryinen, 1984).

The major reason for the gender difference in fasting insulin secretion may be the decreased insulin secretion or C peptide in women with menstrual cycles. Premenopausal women with regular menstrual cycles have enhanced insulin sensitivity when compared to adult men (Jarvis et al, 2013; Park et al, 2003; Yki-Jarvinen 1984) and was proposed to be the cause of reduced incidence of DM2 in premenopausal women (Park et al, 2003; Yki-Jarvinen 1984). After menopause,

there is increase in body fat mass, circulating triglycerides, LDL cholesterol, inflammatory markers and free fatty acids (Carr, 2003; Pfeilschifter et al, 2002; Sites et al, 2002).

Table 9.1. Characteristics of C peptide and diabetes-related parameters and their comparison in participants of groups without (I) and with (II+III) FH. As the variables in (II+III) were not normally distributed, log_{10} transformation was done. When they were normally distributed and there was equality of variance, Student t test was done; but when they were not, non parametric Mann-Whitney U test was done (in brackets).

Variables	Comparison of Groups without (I) and with (II+III) FH					
	I Mean±SD Range (n = 16)	(II+III) Mean±SD Range (n = 64)	After log_{10} transformation			
			Shapiro-Wilk test for normality P		Levene's test for equality of variances P	Student t-test (or Mann-Whitney U test) P
			I	(II+III)		
Age (years)	21.88±1.78 18 - 25	21.78±1.95 18 – 25	0.652	<0.001	0.603	(0.990)
BMI (kg/m^2)	20.38±2.60 17.48 - 26.12	21.83±3.56 15.47 – 29.09	0.154	0.059	0.107	0.149
Waist circumfe-rence (cm)	76.41±7.20 68.00 - 92.5	78.87±8.03 64.50 – 99	0.035	0.144	0.464	(0.213)
Fasting Glucose (mmol/L)	4.74±0.416 3.97 – 5.49	4.93±0.543 4.05-6.70	0.442	0.121	0.312	0.206
2 hour OGTT Glucose (mmol/L)	5.21± 1.06 3.26 - 7.27	5.53±1.40 2.77 - 9.76	0.759	0.239	0.583	0.444
Fasting C Peptide (nmol/L)	0.517±0.129 0.35 - 0.78	0.615±0.20 0.33 – 1.19	0.557	0.204	0.282	0.076
Fasting Insulin (pmol/L)	23.85±6.87 13.26 - 37.32	40.49±18.87 12.24 -106.14	0.943	0.06	0.282	(<0.001)
Total Cholesterol (mmol/L)	4.26±0.619 3.42- 5.26	4.72±0.842 3.44 – 6.89	0.255	0.026	0.329	(0.065)
Triglycerides (mmol/L)	0.764±0.184 0.52 – 1.25	0.925±0.428 0.42-2.72	0.636	0.068	0.034	(0.224)
LDL Cholesterol (mmol/L)	2.48±0.534 1.48 – 3.81	2.97±0.749 1.89 – 5.05	0.700	0.147	0.279	0.011
HDL Cholesterol (mmol/L)	1.43±0.388 0.88 – 2.2	1.31±0.352 0.65-2.25	0.654	0.804	0.629	0.270

Fig. 9.1. Scatter diagram of fasting insulin and C Peptide in the total sample. Log_{10} transformation converted the positively skewed fasting insulin and C Peptide to normal distribution for calculation of Pearsons Correlation coefficient (r = 0.648).

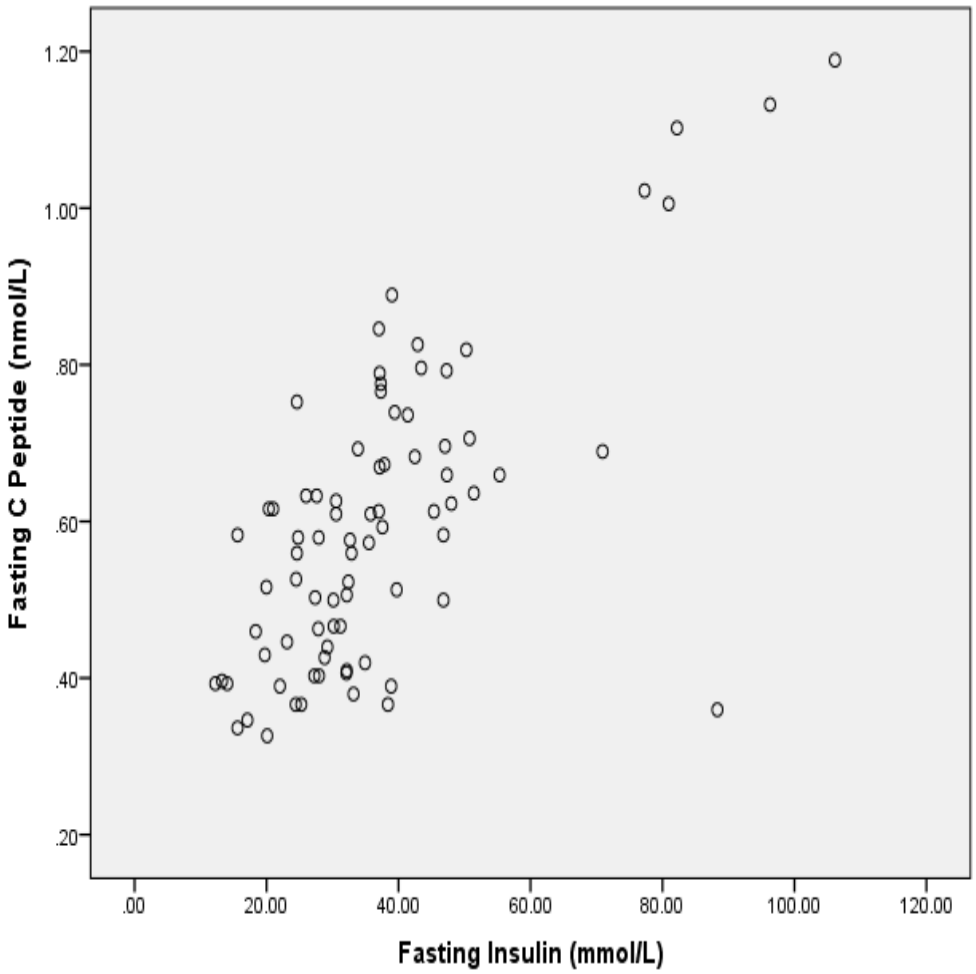


Table 9.2. Comparison of fasting C Peptide in groups I, II and III. As groups II and III were positively skewed, $\log_{10}$ transformation was done followed by one way ANOVA and post hoc pair comparison in conjunction with Tukey HSD and Bonferroni correction.

Groups		**I** (n = 16)	**II** (n = 35)	**III** (n = 29)
Mean±SD		0.517±0.129	0.614±0.169	0.617±0.235
Median		0.488	0.613	0.609
Mode		0.35	0.37	0.39
95% CI of mean		0.448 – 0.586	0.556 – 0.672	0.527 – 0.706
Shapiro Wilk test, P ($\log_{10}$ transformed)		0.557	0.504	0.174
Test of homogeneity of variances ($\log_{10}$ transformed)		Levene statistic = 2.541 (P = 0.085)		
Range		0.35 - 0.78	0.33 – 1.02	0.34 – 1.19
ANOVA (One way)		F ratio = 1.644 (P = 0.200)		
Post hoc pair comparison (with equal variance)	**Tukey HSD**	I and II, P 0.187 II and III, P = 0.309 I and III, P = 0.952		
	Bonferroni correction	I and II, P 0.243 II and III, P = 0.434 I and III, P = 1.000		

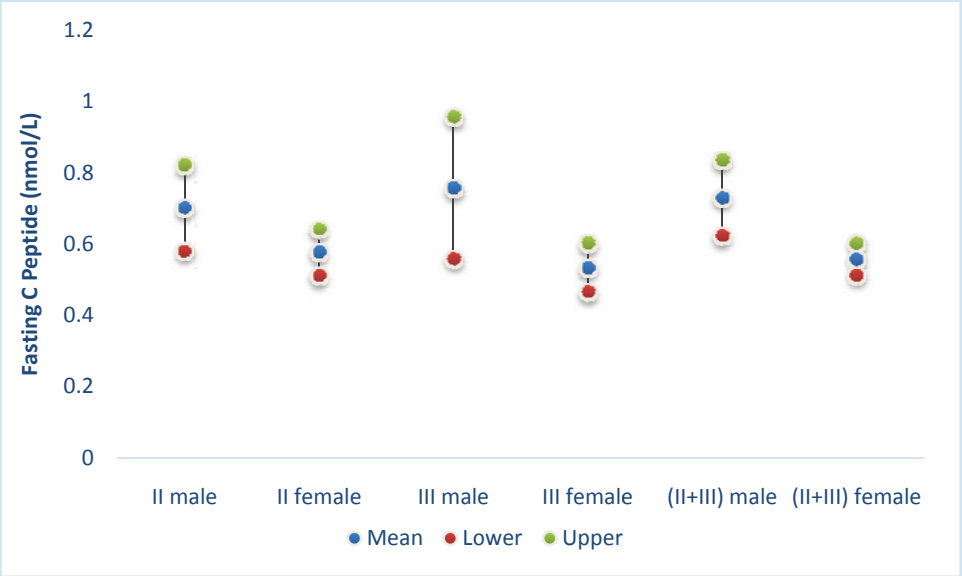
Table 9.3. Comparison of fasting C peptide (nmol/L) between familiality groups I, II and III in males (n = 26) and in females (n = 54) separately (by one way ANOVA and 95% CI of mean), and between males and females in each group (by Student t test). All male and female groups were normal distributed before Log_{10} transformation and Levene's test showed homogeneity of variances.

Groups	**Male fasting C peptide**			**Female fasting C peptide**		
	I(m) n = 4	**II(m) n = 11**	**III(m) n = 11**	**I(f) n = 12**	**II(f) n = 24**	**III(f) n = 18**
Mean ±SD	0.485 ±0.182	0.700 ±0.179	0.755 ±0.297	0.528 ±0.115	0.575 ±0.153	0.532 ±0.138
95% CI of mean	0.195 – 0.775	0.580 – 0.820	0.556 – 0.955	0.454 – 0.601	0.510 – 0.639	0.463 – 0.601
Shapiro Wilk, P	0.136	0.120	0.302	0.420	0.485	0.164
Test of homogeneity of variances	Levene statistic = 2.176, P = 0.136			Levene statistic = 0.816, P = 0.448		
One way ANOVA	F = 1.905; P = 0 .172			F = 0.678; P = 0.512		

Table 9.4. Comparison of fasting C peptide male and female in group I, II, III and (II+III) by Student's t test (Mann Whitney U test).

Groups	I male (n = 4)	I female (n = 12)	II male (n = 11)	II female (n = 24)	III male (n = 11)	III female (n = 18)	(II+III) male (n = 22)	(II+III) female (n = 42)
Mean ±SD	0.485 ± 0.182	0.528 ± 0.115	0.700 ± 0.179	0.575 ± 0.153	0.755 ± 0.297	0.532 ± 0.138	0.728± 0.241	0.556± 0.147
Range	0.35 – 0.75	0.39 – 0.78	0.50 – 1.02	0.33 – 0.89	0.36 – 1.19	0.34 – 0.79	0.36 – 1.19	0.33 – 0.89
95% CI of mean	0.195 – 0.775	0.454 – 0.601	0.580 – 0.820	0.510 – 0.639	0.556 – 0.955	0.463 – 0.601	0.621 – 0.834	0.511 – 0.602
Shapiro Wilk test, P	0.136	0.420	0.120	0.485	0.302	0.164	0.282	0.082
Levene's test P	0.318		0.630		0.005		0.009	
Student's t test (Mann Whitney U test)	0.590		0.041		(0.031)		(0.007)	

Fig. 9.2. 95% confidence intervals of mean of fasting C peptide in the male and females samples of groups II, III and (II+III).



CHAPTER 10

Calculation of the Reference Interval of Total sample population and Partitioned groups of Fasting Insulin and Fasting C peptide

10.1. Abstract

The baseline reference interval of young adult population between the ages of 18 - 25 years excluded the influence of growth phase upto 18 years and the environmental effects after 25 years. When the sample number was 120 or above, the non parametric method of 95% reference interval calculation was done without any transformation. When the sample number was below 120, but above 30, parametric method with robust calculation was used to calculate 95% reference interval. When the sample number was below 30, non parametric range was used as the reference interval. The **95% reference intervals for fasting insulin** were as follows: Total fasting insulin: 12.6 - 92.9 pmol/L (non parametric method); Group I (male and female): 8.5 - 37.3 pmol/L (non parametric range); Group II male 19.9 - 80.9 pmol/L (non parametric range); Group II female: 12.8 - 52.2 pmol/L (parametric); Group III male: 20.9 - 106.1 pmol/L (non parametric range); Group III female: 12.8 - 90.9 pmol/L (parametric). The **95% reference intervals for fasting C peptide** were as follows: Total fasting C peptide: 0.3 – 1.1 nmol/L (parametric method); Group I (male and female): 0.3 – 1.2 nmol/L (non parametric range); Group II male 0.5 – 1.02 nmol/L (non parametric range); Group II female: 0.3 – 0.8 nmol/L(non parametric range); Group III: 0.3 – 1.2 nmol/L (non parametric range); Group III female: 0.4 – 1.1 nmol/L (non parametric range).

10.2. Introduction

The data generated in the laboratory and other clinical data collected from a patient can be interpreted by comparison with the reference data to arrive at a diagnosis of a disease. The interpretation of quantitative biochemistry laboratory data is a case of decision making by comparison. Reference values are required for all clinical laboratory tests, not only from healthy individuals but also from patients with a clinically suspected disease.

Some conditions are required to make the comparison of patient's laboratory results with reference values possible and valid (IFCC 1987-1991; Mardia 1980; Solberg & Grasbeck, 1989).The population from which reference

data is collected should be defined. All the groups of reference individuals should be clearly defined with inclusion and exclusion criteria. Conditions in which the sample were obtained and processed for analysis should be known.All quantities compared should be of same type.All laboratory results should be produced with the use of adequately standardized methods under sufficient analytical quality control.The diagnostic sensitivity and specificity, prevalence and clinical costs of misclassification should be known for all lab tests used. From the parent population, who fulfill the selection criteria, random sampling is the best method for selecting reference individuals. But strict random sampling is not possible due to practical reasons. Therefore, using the best reference sample that can possibly be obtained after all practical considerations have been taken into account, is necessary (Horowitz, 2012).

10.2.1. Partitioning the sample

It is necessary to have separate reference values for sex, age group and other criteria. The number of criteria for partitioning the reference interval should be kept small and significant, so that sufficient sample sizes for valid statistical estimate must be obtained. Several analytes vary significantly among different age and gender groups, so age and sex are most frequently used criteria for sub grouping. Other examples of partitioning criteria to be used for possible sub grouping arethe genetic factors; the race, ethnic, blood and HLA groups**;** physiological factors such as stage of menstrual cycle, pregnancy, physical condition,and factors such as socio economic, environmental and chronobiological states (Horowitz, 2012).

10.2.2. Sample collection and Data generation

The step of sample collection is essential to minimize bias and variation, standardization of pre analytical preparation of individuals before sample collection, sample collection itself and handling of sample before analysis, etc. are necessary. Venipuncture and skin puncture are standardised procedures for blood sample collection (Grasbeck & Alstrom, 1981). After sample collection its analysis is very important. Same analytical method should be used to ensure comparability between reference and observed value. In the analytical procedures the essential components required specification are: Analysis method, including information on

equipment, reagents, calibration type of raw data and calculation methods; quality control; reliability and validity criteria. The specification and validity of the tests should be so clear that another investigator can reproduce the study.

10.2.3. Statistical treatment of Reference Interval

Statistical analysis of reference interval calculations were done for partitioning the reference values, analysis of sample distribution and calculation of reference intervals (Harris & Boyd, 1995; IFCC, 1987; Solberg & Grasbeck, 1989).

10.2.3.1. Partitioning of the Reference data of Fasting Insulin and C peptide

Various influences and correlation of the parameters for reference interval calculation are analyzed for partitioning and to understand the relationship of the parameter. In this study, the major influences of fasting insulin and fasting C peptide were the type 2 diabetes mellitus family history, gender differences, age, and environmental influences. The influences of age, environmental influences and influence of growth phase are restricted in the study by selecting the sample population of male and female between 18 - 25 years of age. Biochemical cut off values of laboratory data are also used to exclude individuals with family history of obesity, liver dysfunction, abnormal lipid profile and other clinical secondary influences.

After exclusion of these parameters, the data was partitioned according to family history of type 2 diabetes mellitus and according to gender.

The various type 2 diabetes mellitus, insulin and C peptide related parameters were also analysed to identify the important influences on these parameters. In the earlier chapters of this dissertation, they were identified and analysed. A strong correlation was identified with post glucose load OGTT insulin, C peptide and fasting triglyceride.

Significant differences in the partitioned group were analysed by parametric and non parametric statistical methods. Before choosing parametric methods, it important to verify various aspects of sample distribution by a number of methods, such as Sharipo Wilk method. The parametric statistical methods used in this study were Student's t test and one way ANOVA. The non parametric

methods were Mann-Whitney U test and Kruskal-Wallis tests (IFCC, Mardia 1980; Solberg 1986)

10.2.3.2. Analysis of Sample Distribution

Sample distribution can be analysed visually and by calculating distribution statistic. The visual method of data analysis was done by histogram, Q-Q plot and Box-Whisker plot. The distribution statistics was calculated by Shapiro-Wilk, D'Agostino and Anderson-Darling methods. While Anderson-Darling methods were based on the distribution of the data in relation to Gaussian distribution, D'Agostino methods rely on the skewness and kurtosis character of the sample distribution (IFCC, 1987; Horowitz, 2012).

When sample distribution was not Gaussian or when the statistic was too low, data transformations were done by $\log_{10}$, ln (natural log) and Box-Cox methods.

10.3. Aim of Study in this section

Calculation of reference intervals of the total and partitioned data of fasting insulin and fasting C peptide.

10.4. Methods and Materials

Baseline reference intervals of young adult population between the ages of 18 - 25 years excluded the influence of growth phase upto 18 years and the environmental effects after 25 years. Strict clinical criteria and cutoff levels of a number of clinical biochemistry parameters were used for inclusion and exclusion criteria to reduce secondary influences on fasting insulin and fasting C peptide (Refer Chapter 2, General Methods).

The following methods for calculation of total sample population baseline reference intervals and partitioned sample reference interval were from the recommendations given by IFCC and from the articles on Reference intervals in

Tietz Textbook of Clinical Chemistry (IFCC, 1987; Horowitz, 2012; IFCC, 1987; Solberg & Grasbeck, 1989; Harris & Boyd, 1995).

When the sample number was above 120, the non parametric method of 95% reference interval calculation was done without any transformation. This was done by calculating the 95% middle centile values and leaving 2.5% at both ends. When the sample number is 120, 95% reference interval removes 3 samples from upper and lower range.

When the sample number was below 120, but above 30, parametric method with robust calculation was used to calculate 95% reference interval. The parametric methods require Gaussian distribution of the sample, different methods of transformation were used to convert a non-Gaussian distribution to Gaussian distribution, preferably with high statistic by Shapiro-wilk, D'Agostino and Anderson-Darling methods. Anderson-Darling method works well for analysis of the low sample number distribution. For 95% reference interval calculations we preferred Box-Cox transformations, as the transforming criteria was variable.

When the sample number was below 30, non parametric range was used to calculate 95% reference interval. This method requires careful analysis of two or three data points near lowest and highest range points. This was to reduce errors at critical lower and upper limits of reference interval. Software used for the study were SPSS, Minitab and MEDCALC (trial versions).

When the sample number was above 120 and when non parametric method was used, Bootstrap sampling was done to make the reference interval close to population reference interval. The 90% confidence interval of the upper and lower limits of the reference interval was also calculated. When parametric methods were used to calculate the 95% reference interval, Robust method of calculations were used to get better values for the reference interval.

10.5. Results and Discussion

10.5.1 . Reference Interval Calculations of Fasting Insulin

The population reference interval of the baseline population without the influence of growth phase below 18 years and without the environmental influence after 25 years, were calculated. As the sample number was above 120, 95% reference interval was calculated by non parametric bootstrap sampling method.

The analysis of sample distribution showed a positively skewed distribution by histogram (Fig.10.1A). The quantile - quantile (Q-Q) plot (Fig. 10.1B) showed a large deviation from Gaussian distribution ($P < 0.005$) (Table 10.1A). The non parametric method excluded three samples from either end for calculating the middle 95% reference interval for sample size of 120. Bootstrapping did repeated sampling so that the reference interval was close to the population reference interval. The calculated non parametric 95% reference interval was from 12.7 - 92.9 pmol/L (Table 10.1B.). The 90% confidence interval for the lower limit of reference interval was 8.5 - 14.0 pmol/L and for the upper limit was 75.0 - 106.1 pmol/L.

The total sample was partitioned into group I, II and III according to the family history of type 2 Diabetes Mellitus. Group II and III was further sub partitioned according to gender. There was no gender difference in group I. As the total sample number of group I was lower than 30 (n = 23), non parametric range was used to calculate the 95% reference interval. The range of fasting insulin in group I was 8.5 - 37.3 pmol/L. The sample in lower and upper limit was repeatedly cross verified for errors of estimation and information bias (Table 10.1B).

The parametric reference interval was calculated from mean and SD. In group I the 95% reference interval was found to be 8.5 - 37.5 pmol/L by parametric robust method of calculation. The 90% confidence interval for upper limit of reference interval ranges from 32.7 - 41.9 pmol/L. The 90% confidence interval for lower limit of reference interval could not be calculated due to the negative values obtained in the calculation and low sample number (Table 10.1B; Fig.10.2)

Group II was partitioned into males and females and the 95% reference intervals were calculated separately. Group II male sample was less than 30 (n = 22), therefore non parametric method was used by calculating the range. Reference interval was taken as the range of fasting insulin from 19.9 to 80.9 pmol/L. Group II male sample could not be transformed to Gaussian distribution by $\log_{10}$, natural logarithm (ln) or by Box-Cox transformation (Fig.10.3). Therefore, parametric methods could not be used for calculating reference interval.

Group II female sample (n = 38) was used for 95% reference interval calculation by parametric method using Robust calculation (Table 10.1B.). The reference interval calculated was 12.8 - 52.2 pmol/L. The 90% confidence interval of the lower limit of reference interval was 8.5 - 17.8 pmol/L and this were found

to be a wide range due to the low sample number. The 90% confidence interval of the upper limit of reference interval was 47.6 - 56.8 pmol/L. The non parametric range was 13.7 - 55.3 pmol/L.

In group III male the sample number was low (n = 19), there for non parametric range was used to calculate 95% reference interval. The range was 20.9 - 106.1 pmol/L. Group III female samples had a sample number above 30 (n = 34), both parametric and non parametric method was used to calculate 95% reference interval (Fig.10.4). The parametric method using Robust sampling gives a 95% reference interval of 12.8 - 90.9 pmol/L. The 90% confidence interval of the lower limit of reference interval was 8.9 - 17.9 pmol/L and the upper limit of reference interval was 74.6 - 105.9 pmol/L. The non parametric range was calculated as 12.2 - 97.6 pmol/L.

10.5.1.1. Reference Interval chosen for Fasting Insulin

1. Total fasting insulin sample: selected method non parametric with bootstrap sampling; calculated reference interval 12.6 - 92.9 pmol/L.
2. Group I (male and female) fasting insulin sample: selected method non parametric range; calculated reference interval: 8.5 - 37.3 pmol/L.

   As the sample was well distributed parametric method with robust calculation was used and the reference interval obtained was almost same as reference interval by non parametric method: 8.5 - 37.5 pmol/L.
3. Group II male fasting insulin sample: selected method non parametric range; calculated reference interval 19.9 - 80.9 pmol/L.

   Parametric method was not used as the sample distribution had lower distribution statistic.
4. Group II female fasting insulin sample: selected method parametric with robust sampling; calculated reference interval 12.8 - 52.2 pmol/L.
5. Group III male fasting insulin sample: selected method non parametric range; calculated reference interval 20.9 - 106.1 pmol/L.
6. Group III femalefasting insulin sample: selected method parametric with robust sampling; calculated reference interval 12.8 - 90.9 pmol/L.

Our method of calculation of baseline reference interval in young adults aged 18 - 25 years gave a 95% reference interval of 12.7 - 92.9 pmol/L.The lower, upper limits of reference interval in our study showed that it is a baseline reference interval which is likely to increase with age and environmental influence.

Partitioned groups are better representatives of baseline reference interval. It was showed that family history of type 2 diabetes mellitus up to grandparents and gender difference in the probands of participants of the study influence reference interval. There for partitioned reference interval (Table 10.1B.) can be more useful for clinical interpretation of fasting insulin levels.

10.5.1.2. Comparison of the earlier reported reference interval of Fasting Insulin with the above reporting values

The reference interval of fasting insulin in an adult sample population reported earlier were 2 – 25 μIU/ml (12 – 150 pmol/L) (Burtis et al, 2012), in another report it was 2 – 20 μIU/ml (12 – 120 pmol/L) (Longo et al, 2012) and in third report it was 1.9 – 23 μIU/ml (11.4 – 138 pmol/L) (Ultrasensitive insulin, 2005). Our reporting value for baseline reference interval of fasting insulin is 12.7 – 92.9 pmol/L (2.1 – 15.9 μIU/ml). The major difference between the reported total fasting insulin reference interval and our reporting reference interval is the upper limit of the reference value. Our reference value for total sample population is a baseline reference value. A baseline reference value is a value obtained after excluding environmental influences and other secondary clinical influences in a baseline population. The baseline population here are young adults 18 – 25 years of age from a rural sample population. This age group excludes the influence of growth phase upto 18 years of age and environmental build up after 25 years of age.

The partitioned reference interval has not been reported earlier in our knowledge. Group I without family history of type 2 diabetes upto grandparents is the most basic of the baseline partitioned reference interval. Group II and III are moderately or highly predisposed genetically.

The lower and upper limit of fasting insulin were higher in males when compared to that of females. The lower limit of reference interval of group I, group II females and group III females were almost same at 12 pmol/L.

10.5.2. Reference Interval CalculationsOf Fasting C Peptide

Total fasting C peptide distribution was positively skewed (Fig. 10.5A) and after Box-Cox transformation the distribution become Gaussian (Fig. 10.5B). The Anderson-Darling method distribution analysis also showed a Gaussian distribution, with P = 0.158 (Table. 10.2A). As the sample number was less than 120, parametric method with robust calculation was used for reference interval estimation. The 95% reference interval was 0.32 - 1.11 nmol/L. Non parametric method with bootstrap sampling was used for 95% reference interval calculation of 80 C peptide samples. The calculated 95% reference interval was 0.34 – 1.12 nmol/L (Table 10.2B).

In group I males and females were clubbed together (n = 16). The sample showed a good Gaussian distribution after Box-Cox transformation (P = 0.83) (Table 10.2A; Fig.10.6). As the sample number was low, non parametric range was used for 95% reference interval calculation and the reference interval was 0.33 - 1.19 nmol/L. The 95% reference interval by parametric method with robust calculation was 0.32 - 0.97 nmol/L. The 90% confidence interval of the lower limit of reference interval was 0.3 - 0.36 nmol/L and the upper limit of reference interval was 0.7 - 1.3 nmol/L (Table 10.2B).

In group (II+III) male samples were clubbed together, as there was no difference between fasting C peptide levels. After Box-Cox transformation this sample (n = 22) gave a Gaussian distribution (Anderson-Darling test, P = 0.187) (Table 10.2A; Fig.10.7). Non parametric range was chosen for 95% reference interval calculation and it was 0.34 - 1.19 nmol/L (Table 10.2B).

Group (II+III) female could not be partitioned according to family history of type 2 diabetes mellitus up to grandparents as the fasting C peptide levels were similar. As the sample number was above 30 (n = 42) the parametric robust method was used for calculating 95% reference interval and the 95% reference interval was 0.32 - 1.15 nmol/L. The 90% confidence interval of the lower limit of reference interval was 0.3 - 0.4 nmol/L and the upper limit of reference interval was 1.0 - 1.3 nmol/L. As the sample number was above 40 (n = 42) 95% reference interval was also calculated by non parametric bootstrap sampling and it was 0.35 - 1.12 nmol/L (Table 10.2B; Fig.10.8).

10.5.2.1Reference interval chosen for fasting C peptide

1. Total fasting C peptide sample: selected method parametric with robust sampling; calculated reference interval 0.3 – 1.1 nmol/L.

2. Group I fasting C peptide sample: selected method non parametric range; calculated reference interval 0.3 – 1.2 nmol/L.
3. Group II male fasting C peptide sample: selected method non parametric range sampling; calculated reference interval 0.5 – 1.02 nmol/L.
4. Group II female fasting C peptide sample: selected method non parametric range; calculated reference interval 0.3 – 0.8 nmol/L.
5. Group III male fasting C peptide sample: selected method non parametric range; calculated reference interval 0.3 – 1.2 nmol/L.
6. Group III female fasting C peptide sample: selected method non parametric range; calculated reference interval 0.4 – 1.1 nmol/L.

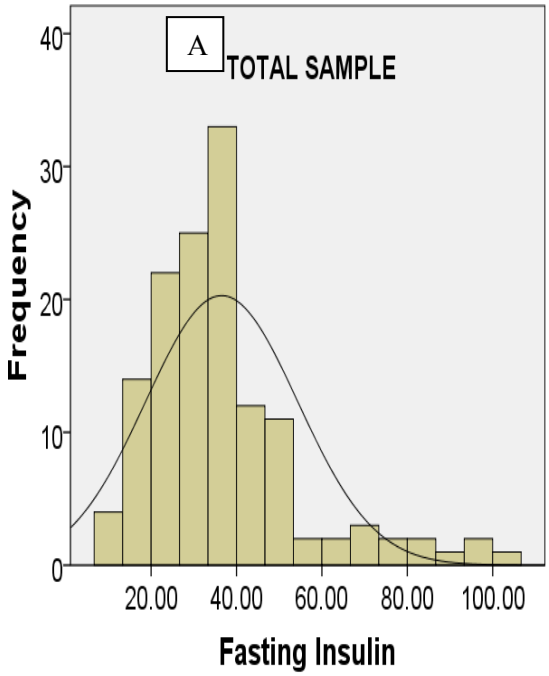
As the lower limit of reference interval was similar around 0.32 - 0.34 nmol/L and because reference interval of all the four group were overlapping, the reference interval of the total sample by parametric robust calculation was chosen as the common reference interval for fasting C peptide.

10.5.2.2. Comparison of the earlier reported reference interval of Fasting C peptide with the above reporting values

The reported reference interval of fasting C peptide was 0.78 – 1.89 ng/ml (0.26 – 0.62 nmol/L) (Brutis and Ashwood, 1999). Our reporting total fasting C peptide reference interval is 0.3 – 1.1 nmol/L. The upper limit of fasting C peptide was much higher in our studies reported above. The higher upper limit of fasting C peptide is because of the robust and active secretion of fasting C peptide in young non diabetic adults from a rural sample population. As age increases and in IGT and diabetes mellitus there is decrease in insulin secretion and the upper limit of fasting C peptide is decreased. This is the cause for confounding of insulin secretion by beta cell dysfunction. Therefore, our higher upper limit of fasting C peptide reference interval represents the healthy baseline value.

Group I, II male and III male samples have higher upper limit of fasting C peptide. In group II female the upper limit of fasting C peptide is 0.8 nmol/L. This represents a decreased insulin secretion due to increased insulin sensitivity in young adult women. In group III female the fasting C peptide higher at 1.1 nmol/L. Except in group II female, the upper limit of reference interval of fasting C peptide is 1.02 – 1.2 nmol/L.

Fig.10.1. Distribution characteristics of fasting insulin in total sample population by Histogram (A) and Anderson – Darling probability plot (B).



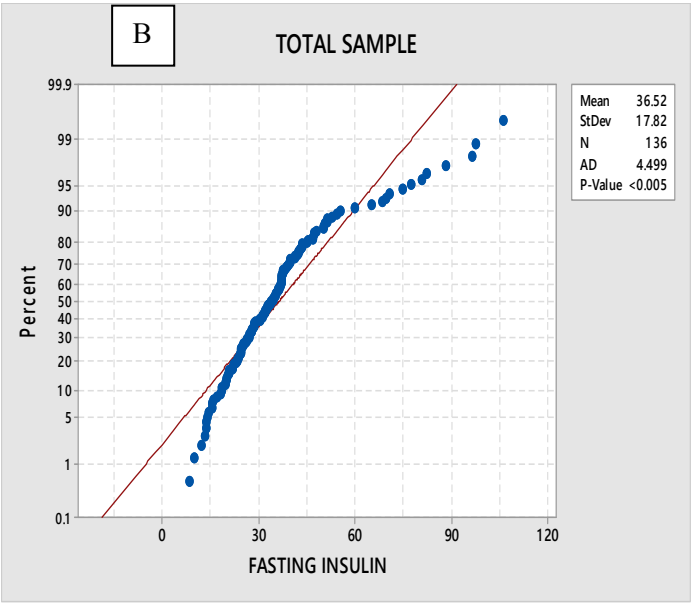


Table 10.1A. Distribution characteristics after transformation of fasting Insulin in the total sample and in various groups and their ranges.

Fasting Insulin in Total sample and Partitioned groups	Range	95% CI of mean	Inter Quartile Range	Shapiro-Wilk test P	D'Agostino-Pearson test P	Anderson-Darling test P
Total sample n = 136	8.46 – 106.14	33.49 – 39.54	17.76	<0.0001	<0.0001	<0.005
Group I n = 23	8.46 – 37.32	18.79 – 25.22	0.75	0.76	0.798	0.84
Group II male n = 22, Box - Cox λ = (-0.23)	19.86 – 80.94	32.97 – 46.63	12.18	0.24	0.84	0.13
Group II Female n = 38	13.68 – 55.32	29.61 – 35.89	11.91	0.621	0.84	0.47
Group III male n = 19, Box-Cox λ = (-0.7)	20.88 – 106.14	36.02 – 60.45	0.02	0.551	0.984	0.47
Group III female n = 34, Box-Cox λ = (0.35)	12.24 – 97.56	36.17 – 49.41	0.7	0.81	0.94	0.674

Table 10.1B. Reference intervals of Fasting Insulin in the total sample and in various groups calculated by parametric and non parametric methods.

Fasting Insulin in Total sample and Partitioned groups	95% Reference interval (pmol/L) (90% CI of lower and upper limit of Reference Interval)	
	Parametric Robust method	Non-parametric method
Total sample n = 136	---	12.67 - 92.91 (8.46 - 14.04: 75.00 - 106.14) By Bootstrap sampling
Group I n = 23	8.5 - 37.3 (--- ; 32.7 - 41.9)	8.46 – 37.32 Range
Group II male n = 22 Box-Cox λ = (-0.23)	---	19.86 – 80.94 Range
Group II Female n = 38	12.8 - 52.2 (8.48 - 17.76; 47.61 - 56.76)	13.68 – 55.32 Range
Group III male n = 19 Box-Cox λ = (-0.7)	---	20.88 - 106.14 Range
Group III female n = 34 Box-Cox λ = 0.35	12.8 - 90.9 (8.87 - 17.93; 74.58 - 105.87)	12.24 - 97.56 Range

Fig.10. 2. Distribution characteristics of fasting insulin in Group I sample by Anderson – Darling probability plot without transformation. An example of low sample number with a good Gaussian distribution (P = 0.843).

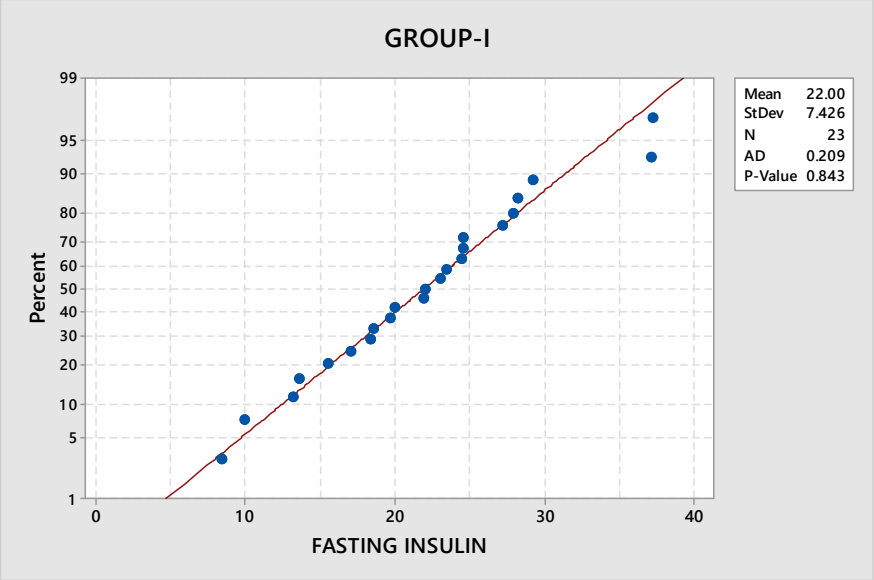
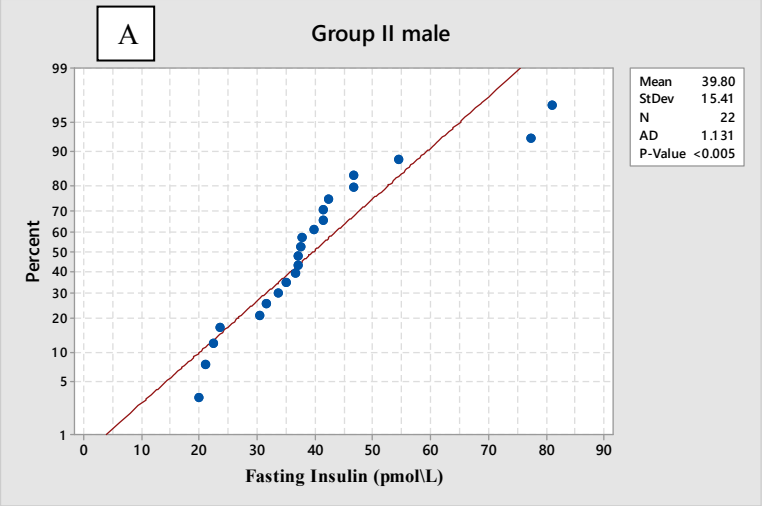


Fig.10.3. Distribution characteristics of fasting insulin in Group II male sample before (A) and after (B) Box-Cox transformation, analysed by Anderson – Darling probability plot. An example of a low sample number group which was weakly transformed to Gaussian distribution (P = 0.125).



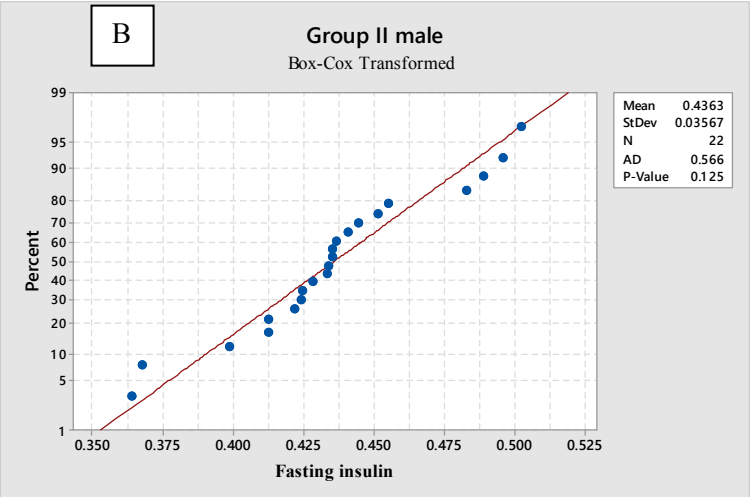
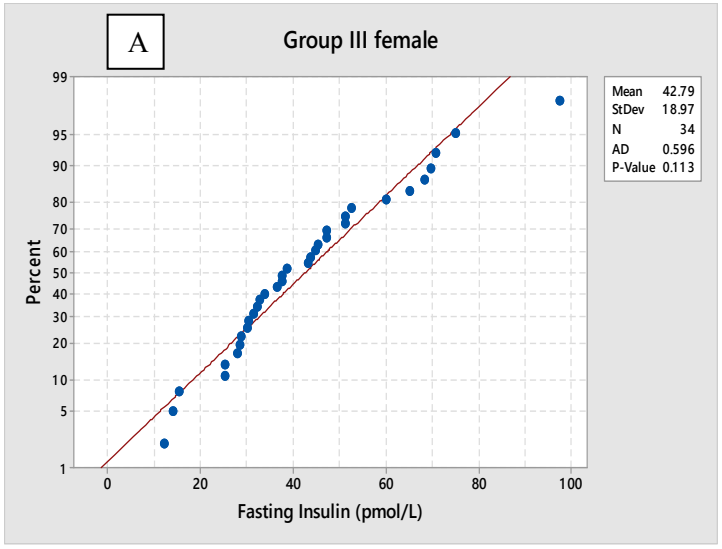


Fig.10. 4. Distribution characteristics of fasting insulin in Group III female sample before (A) and after (B) Box-Cox transformation by Anderson – Darling probability plot. An example of a good Gaussian distribution (P = 0.674) after transformation.



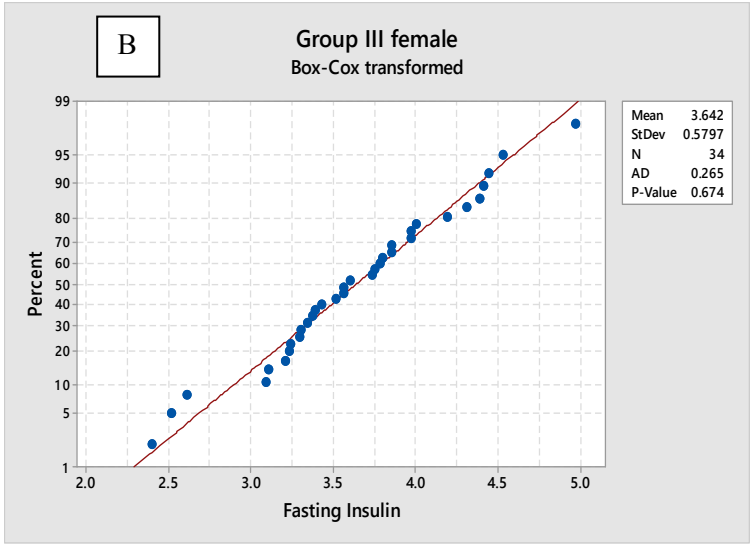
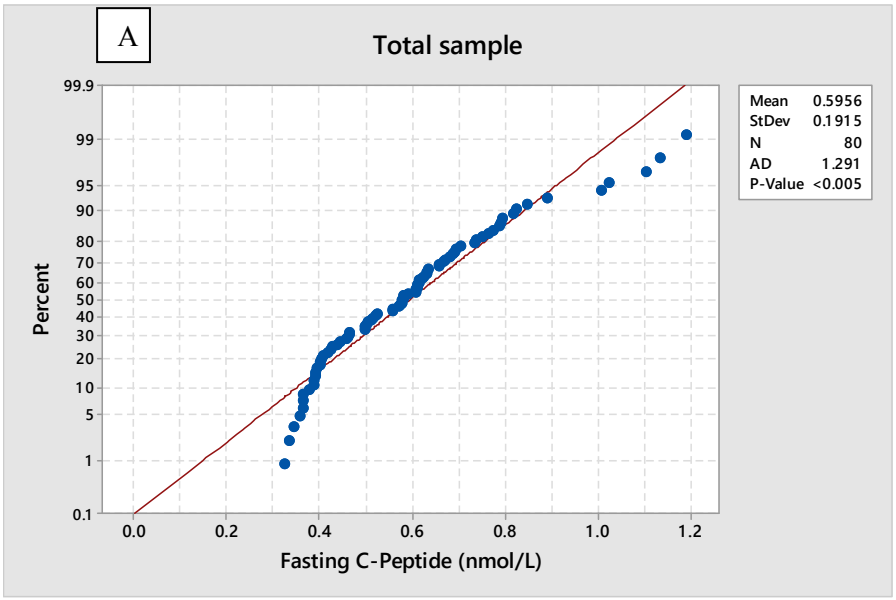


Fig.10.5. Distribution characteristics of fasting C peptide in the Total Sample population by Anderson – Darling probability plot before (A) and after (B) Box-Cox transformation. A positively skewed distribution (P < 0.005) was transformed to Gaussian distribution (P = 0.158).



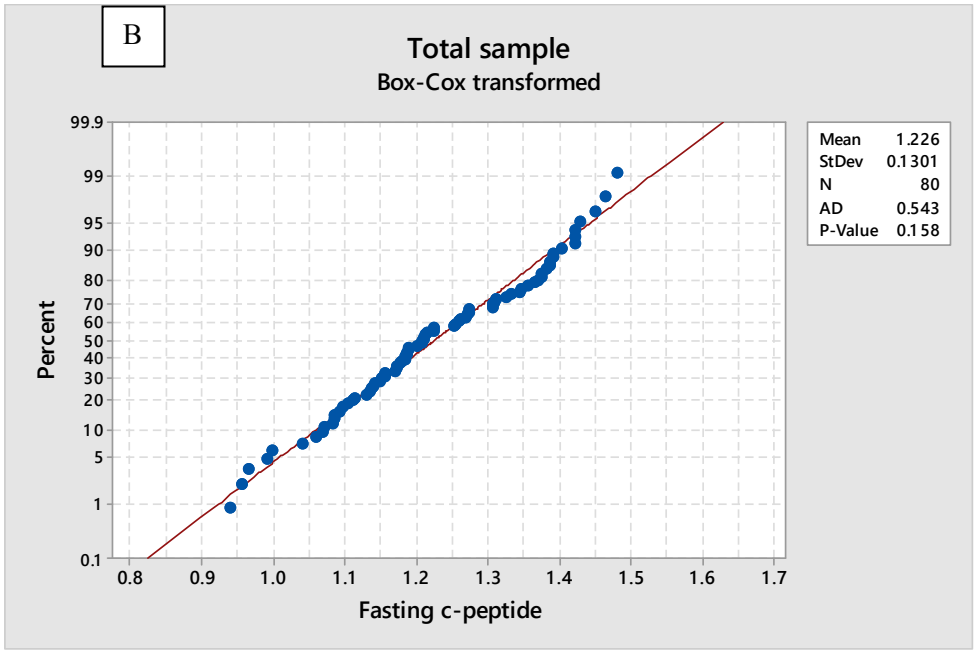
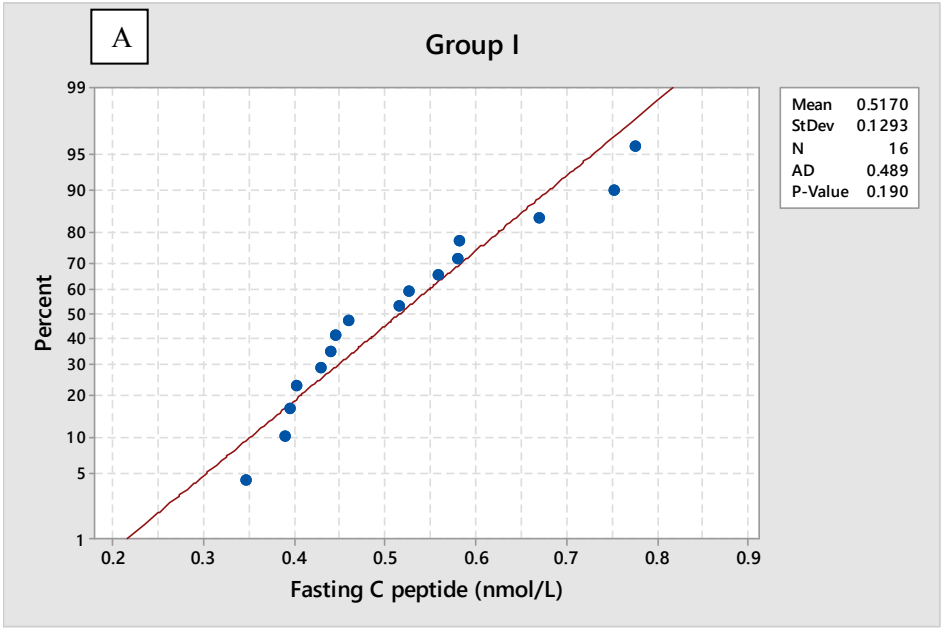


Fig.10.6. Distribution characteristics of fasting C peptide in Group I sample by Anderson – Darling probability plot before (A) and after (B) Box-Cox transformation. A weak Gaussian distribution with low P of 0.190 was transformed to high Gaussian distribution with higher P value of 0.827.



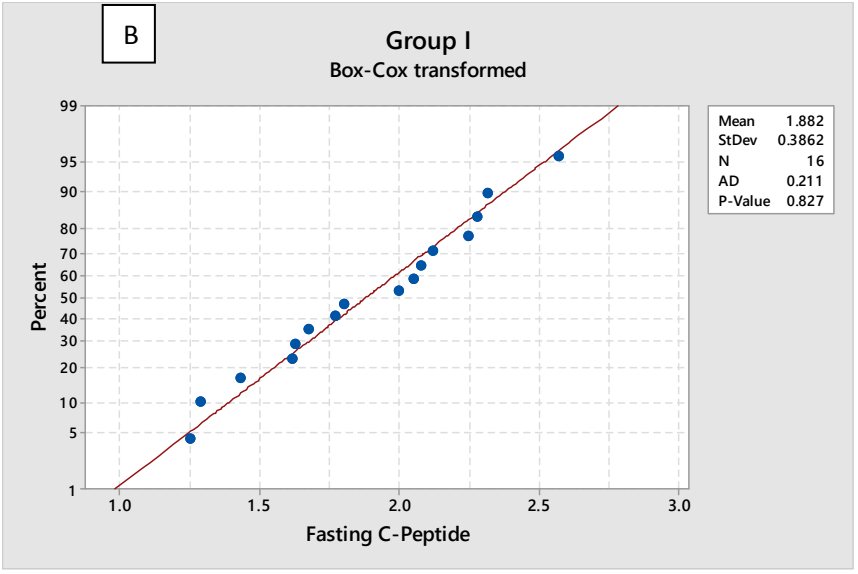
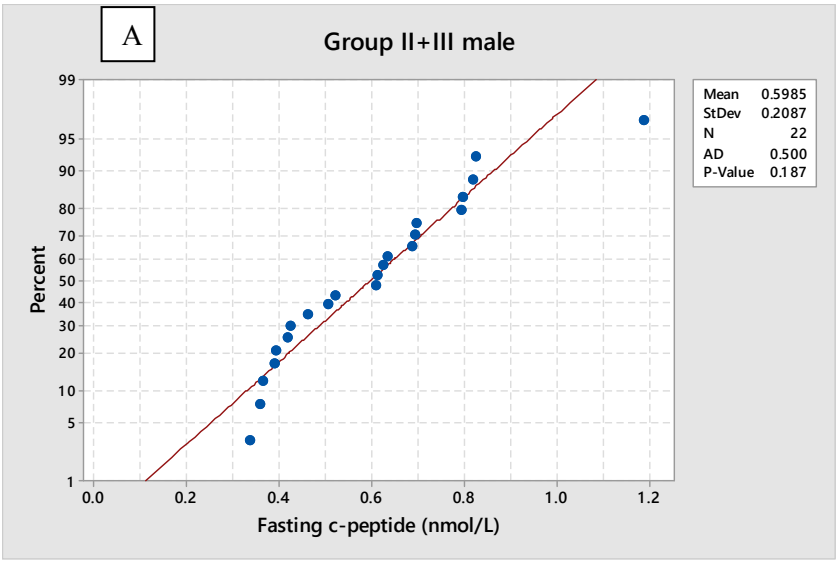


Fig.10.7. Distribution characteristics of fasting C peptide in group (II+III) male sample by Anderson – Darling probability plot before (A) and after (B) Box-Cox transformation. A Gaussian distribution with low P of 0.187 was transformed to Gaussian distribution with higher P value of 0.309.



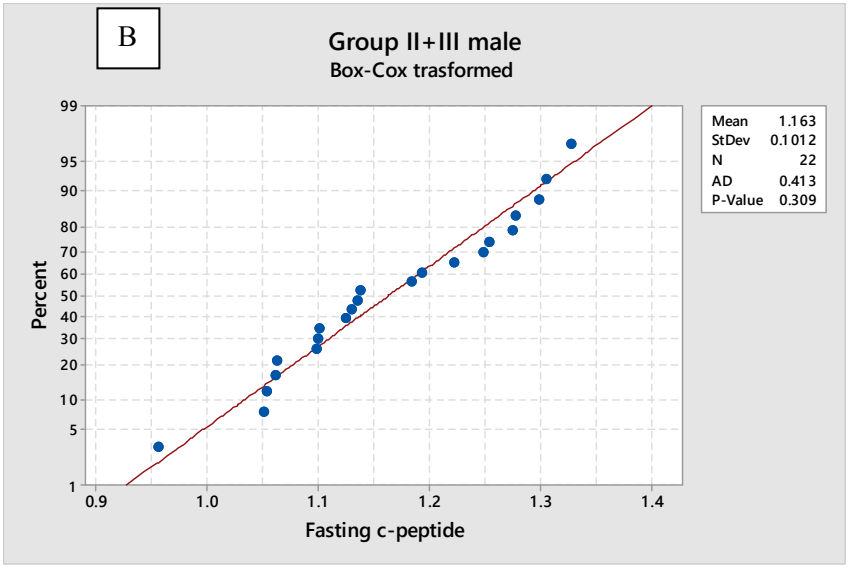
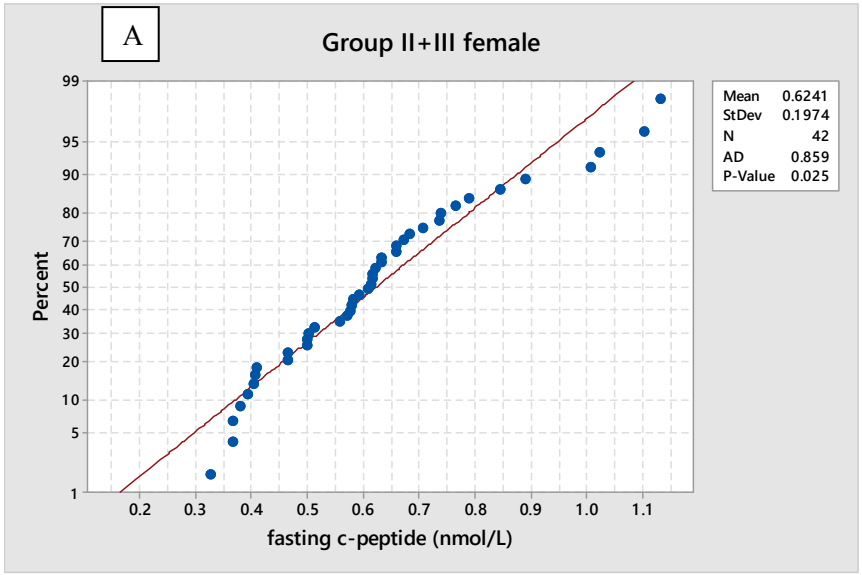


Fig.10.8. Distribution characteristics of fasting C peptide in group (II+III) female sample by Anderson – Darling probability plot before (A) and after (B) Box-Cox transformation. The sample did not have Gaussian distribution (P = 0.025) but had Gaussian distribution after transformation (P = 0.435).



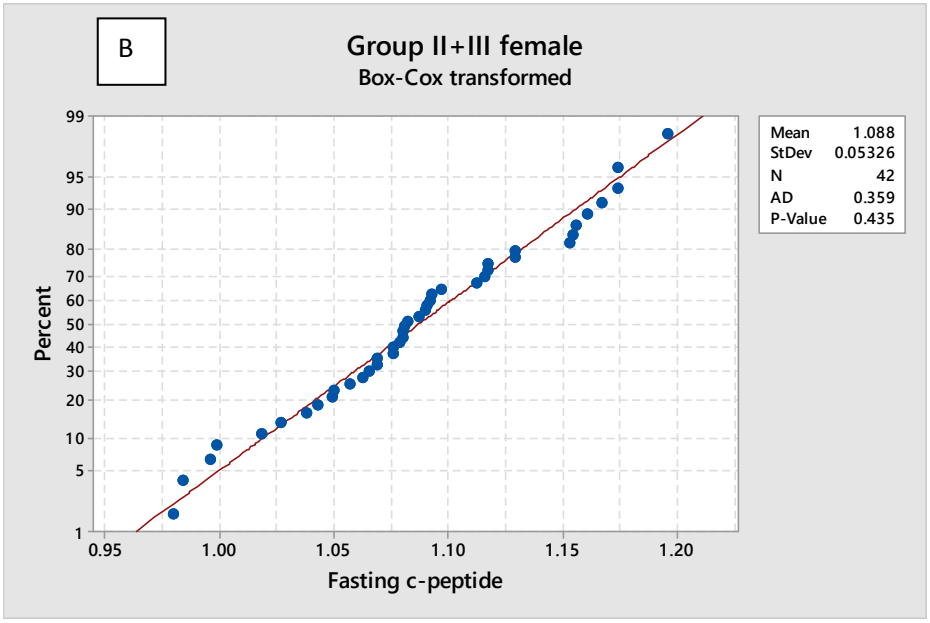


Table 10.2A. Distribution characteristics after transformation of fasting C peptide in the total sample and in various groups and their ranges.

Fasting C peptide in Total sample and Partitioned groups	Mean	Range	95% CI of mean	Inter Quartile Range	Shapiro – Wilk Method P	D'Agostino Method P	Anderson Darling method P
Total sample n = 80	0.59	0.33 – 1.19	0.55 – 0.64	0.64	0.18	0.24	0.158
Group I n = 16	0.52	0.35 – 0.78	0.45 – 0.59	0.17	0.81	0.72	0.83
Group (II+III) male n = 22	0.598	0.34 – 1.19	0.51 – 0.69	0.31	0.001	0.57	0.187
Group (II+III) female n = 42	0.62	0.33 – 1.13	0.56 – 0.69	0.22	0.5	0.93	0.435
Group II male n = 11 Box-Cox λ = (-0.24)	0.699	0.49 – 1.02	0.58 – 0.82	0.21	0.52	0.55	0.74
Group II female (n = 24) Box-Cox λ = 1.21	0.58	0.33 – 0.79	0.51 – 0.64	0.23	0.283	0.51	0.24
Group III male n = 11 Box-Cox λ = (-1.31)	0.76	0.33 – 1.19	0.56 – 0.96	0.64	0.79	0.58	0.786
Group III female n = 18 Box-Cox λ = (-0.21)	0.53	0.36 – 1.13	0.46 – 0.60	0.25	0.714	0.82	0.81

Table 10.2B. Reference intervals of Fasting C peptide in the total sample and in various groups calculated by parametric and non parametric methods.

Fasting C peptide in Total sample and Partitioned groups	**95% Reference interval (pmol/L) (90% CI of lower and upper limit of Reference Interval)**	
	Parametric Robust method	**Non parametric**
Total sample n = 80	0.32 – 1.11 (0.3 - 0.4; 1.0 - 1.3)	0.34 – 1.12 (Bootstrap sampling)
Group I n = 16	0.32 - 0.97 (0.3 - 0.36; 0.7 - 1.3)	0.33 - 1.19 (Range)
Group (II+III) male n = 22	0.3 - 1.27 (0.25-0.36; 1.0-1.6)	0.34 - 1.19 (Range)
Group (II+III) female n = 42	0.32 - 1.15 (0.3-0.4; 1.0-1.3)	0.35 - 1.12 (Bootstrap sampling)
Group II male n = 11 Box-Cox λ = (-0.24)	0.397 - 1.36	0.49 - 1.02 (Range)
Group II female n = 24 Box-Cox λ = 1.21	0.26 - 0.84 (0.18-0.37; 0.78-0.89)	0.33 - 0.79 (Range)
Group III male n = 11 Box-Cox λ = (-1.31)	0.28 - 12.8	0.33 - 1.19 (Range)
Group III female n = 18 Box-Cox λ = (-0.21)	0.31 - 1.35 (0.26 -0.38; 1.04 - 1.7)	0.36 - 1.13 (Range)

Chapter 11

Predisposition and Risk Calculation for Hyperinsulinemia and Increased Insulin Secretion in Young Adults with Family History of Type 2 Diabetes Mellitus

11.1. Abstract

Hyperinsulinemia is associated with family history of type 2 diabetes mellitus. Therefore, hyperinsulinemia indicates increased genetic risk of type 2 diabetes mellitus indicating group II and III with greater genetic predisposition for the disease, when environmental exposures are favorable. In this study, the risk of hyperinsulinemia was calculated by identifying a cutoff level of fasting insulin between the control group I and group II. The midpoint between the 95% CI of mean of fasting insulin of these groups was taken as the cutoff value. With this cutoff value the odds were calculated for groups I, II and III. The odds ratio was estimated for groups II and III with reference to group I for calculating the risk of hyperinsulinemia. This risk increased from group I to II and from group II to III for both males and females. The risk was also higher in males. In the case of fasting C peptide, the levels were increased in males, indicating increased insulin secretion in male the sample. Insulin secretion increased with increased insulin resistance. The odds ratio was higher in group II and III, but group III had lower value than II. But odds ratio of group III male and female samples were not dependable as they showed severe confounding of insulin secretion by β cell dysfunction. Odds ratio for the male samples were higher than that of the female sample, indicating higher insulin resistance and higher risk of type 2 diabetes in males.

11.2. Introduction

There are number of methods for calculating the risk of a disease after getting an exposure. The exposure may be genetic or environmental. Type 2 diabetes is a multifactorial disease with multiple genetic and environmental influences. One such method for calculating the risk for a quantitative clinical biochemistry variable is odds ratio. Odds ratio is the measure of the association between an exposure and an outcome. The odds ratio represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure, expressed as a ratio (Riffenburgh, 2012). Odds ratio is commonly used in case control, cross sectional and cohort studies (Szumilas, 2010).

This study, is a cross sectional study in which the sample population is partitioned into groups of high, low or no exposure. In this study, the exposure is family history or genetic exposure to type 2 diabetes mellitus in parents and grandparents. The second exposure in this study is the gender difference. When there is an exposure, there is a predisposition to the disease, type 2 diabetes mellitus. The two quantitative biochemical variables that were studied were fasting insulin and fasting C peptide. The family history of type 2 diabetes upto grandparents is an exposure that influences fasting insulin (Chapter 7). When there is no influence of family history of type 2 diabetes, the sample was partitioned into group I. In this cross sectional study, therefore, group I was taken as the control. The group coming just after group I was taken as group II, were there was family history in grandparents and not in parents. The cutoff levels were determined between these two groups. Therefore, group II and groups III were compared with that of group I for calculating the odds ratio.

No significant gender difference for fasting insulin was observed in group I. Therefore, group I was not partitioned into males and females (Chapter 8).

There was no significant gender difference observed for fasting C peptide in group I. Therefore, no partitioning was done for fasting C peptide in group I (Chapter 9).

Gender differences were found to influence fasting C peptide. Gender difference in fasting C peptide were seen when there was family history of type 2 diabetes, that is, in group II and III. But family history does not significantly influence fasting C peptide independently. As the lower level were seen in female sample the cutoff was determined between females samples of group II and group I. But for comparison of male samples with group I independent cutoff levels were made between male samples of group II and group I (Chapter 9).

The truth table format of the contingency table is the case in which one variable represents a prediction that a condition will occur and the other variable will predict the outcome, that is, ‘truth’. Four possible situations are named in the table: 1) true positive; when the predicted disease is present, 2) false negative; the event of predicting no disease when disease is present, 3) false positive; the predicted disease is absent, 4) true negative; the event of predicting no disease when disease was absent. In this contingency table, the disease is predicted when there is exposure and the disease is not predicted when there was no exposure.

These two conditions represent the two column of the contingency table. The presence or absence of disease represents the two rows and the total is given in the third row (Riffenburgh, 2012).

The outcome of the exposure to family history of type 2 diabetes in this study is the fasting hyperinsulinemia. In the case fasting C peptide the outcome is the increased fasting C peptide in male gender resulting from increased insulin secretion due to increased insulin resistance in males. Both fasting hyperinsulinemia and insulin resistance are directly or indirectly associated with increased prevalence of type 2 diabetes.

Odds ratios are used to compare the relative odds of the occurrence of the outcome of interest (e.g. diseases, disorders or levels of intermediate markers), given exposure to the ariable of interest (e.g. health characteristic, aspect of medical history) the odds ratio can also be used to determine whether a particular exposure is a risk factor for a particular outcome, and to compare the magnitude of various risk factors for that outcome (Riffenburgh, 2012, Szumilas, 2010).

11.3. Aim of Study in this section

Analysis of predisposition to type 2 diabetes and the risk calculation of hyperinsulinemia and increased insulin secretion by odds ratio. Insulin secretion was represented by fasting C peptide in males and females.

11.4. Materials and Methods

Odds ratio were calculated for fasting insulin and fasting C peptide by the following method. Odds were calculated for group I and group II male by taking the number of individuals above and below the cutoff. The odds of group II male is divided by odds of group I gave the odds ratio.

Similarly, odds ratio was calculated independently for group II and III females by comparing with that of group I (control).

The odds of both male and female groups were also calculated with a common cutoff.

The contingency table in truth table format is given below (Riffenburgh, 2012, Szumilas, 2010):

	Predicted to have disease or is exposed	Not predicted to have disease or is not exposed	Totals
Disease present	True positive (A)	False negative (C)	Yes
Disease absent	False positive (B)	True negative (D)	No
Totals	Predicted Yes	Predicted No	

Where A = Number of exposed cases

B = Number of exposed non-cases

C = Number of unexposed cases

D = Number of unexposed non-cases

$$OR = \frac{a/c}{b/d} = \frac{ad}{bc}$$

$$\frac{(n)exposedcases/(n)unexposedcases}{(n)exposednon-cases/(n)unexposednon-cases} = \frac{(n)exposedcasesX(n)unexposednon-cases}{(n)exposednon-casesX(n)unexposedcases}$$

The interpretation of odds ratio is as follows:

OR = 1, exposure does not affect odds of outcome

OR > 1, exposure associated with higher odds of outcome

OR < 1, exposure associated with lower odds of outcome

The 95% confidence interval (CI) is used to estimate the precision of the OR (odds ratio). A large CI indicates low level of precision of the OR, whereas a small CI indicates a higher precision of the OR. It is important to note however, that unlike the p value, the 95% CI does not report a measure's statistical significance. In practice the 95% CI is often used as a proxy for the presence of statistical significance if it does not overlap the null value (e.g. OR = 1). Nevertheless, it would be inappropriate to interpret an OR with 95% CI that spans the null value as indicating evidence for lack of association between the exposure and outcome.

11.5. Results and Discussion

11.5.1. Determination of cutoff levels of fasting insulin for calculation of odds ratio separately in males and females

Cutoff levels were calculated by comparing the 95% confidence interval of mean of group I and II in males and females (table 11.1). The average of the upper limit of group I and the lower limit of group II was taken as the cutoff levels for fasting insulin. The calculated cutoff values for male samples were 29.1 pmol/L and for female was 27.4 pmol/L (Table 11.2). The odds ratio and the relative risk were calculated separately for the male sample and female sample with reference to group I.

By use of this method the odds ratio of group II and III male samples were 30 and 25, respectively (Table 11.3). But the odds ratio for group II and III females sample were 7.8 and 20.88, respectively (Table 11.3). These results showed that both for group II and III the risk of hyperinsulinemia were higher in males. Hyperinsulinemia is related to family history of type 2 diabetes mellitus in group II and III. Therefore, the higher odds ratio in group II and III indicates the higher risk of type 2 diabetes mellitus in group II and III males. This observation is consistent with higher prevalence of type 2 diabetes mellitus in males. The most significant observation was that the markedly lower risk of hyperinsulinemia in group II females.

11.5.2. Determination of cutoff levels of fasting C peptide for calculation of odds ratio separately in males and females

Cutoff levels were calculated by comparing the 95% confidence interval of mean of group I and II in males and females (Table 11.4). The average of the upper limit of group I and the lower limit of group II was taken as the cutoff levels for fasting C peptide. The calculated cutoff values for male samples were 0.585 nmol/L and for female was 0.55 nmol/L (Table 11.5). The odds ratio and the relative risk were calculated separately for the male sample and female sample with reference to group I.

Insulin resistance is compensated by increase insulin secretion. Insulin resistance and β cell dysfunction are the 2 fundamental defects contributing to pathogenesis of type 2 diabetes mellitus. As insulin resistance increase fasting C

peptide should increase from group I to II and from group II to III. Therefore the odds should increase from group I to III in both male and female samples. But it was observed that both in group III male and group III female the odds decreased when compared to that of group II. From these observations we may assume that insulin secretion increases from group I to II both in males and females, but the increase is very much high in males, indicating increased insulin resistance. But in group III the decrease in odds ratio in both males and females indicates insulin secretory deficiency. This phenomenon of decreasing insulin secretion is confounding an increase in insulin secretion. This leads to conflicting indicators of odds ratio in both male and female. The results indicated that unless and until β cell dysfunction is clinically evaluated independently in insulin resistance. It is not possible to calculate the risk of type 2 diabetes mellitus using C peptide.

11.5.3 Determination of common cutoff levels of fasting insulin in males and females and calculation of odds ratio and relative risk

The cutoff levels between group I and group II female samples were used as the common cutoff level. This is because group II female have lower level of fasting insulin and females before menopause have lower risk of type 2 diabetes mellitus. Therefore group II female were chosen for calculation of cutoff levels. Use of common cutoff levels means groups fasting insulin and their odds ratio are comparable.

When the cut off levels of fasting insulin were common for both male and females it may be possible to combine the odds ratio of male and female samples (Table 11.1.3). By this method both male and female fasting insulin had comparable odds ratio of 20.88 and 19.04, respectively. But there was a significant difference of odds ratio in group II male and females. Group II male odds ratio was 16.07 and group II female odds ratio was 7.8. These results showed significantly lower risk of hyperinsulinemia in group II female.

11.5.4. Determination of common cutoff levels of fasting C peptide in males and females and calculation of odds ratio and relative risk

Interpretation of fasting C peptide odds ratio were complicated by the confounding of insulin secretion by β cell dysfunction. Insulin resistance is expected to increase from group I to II and from group II to group III. We expect

fasting C peptide to increase from group I to II and to group II to III. But fasting C peptide was increased to group I to II in both males and females (Table 11.2.3). But fasting C peptide was decrease from group II to III in both males and females, indicating confounding of insulin secretion by β cell dysfunction. But odds ratio with a common cut off insulin secretion was much higher in males, compared to that of females in group II. But in group III the falling odds ratio was more significant in males. But prevalence of type 2 diabetes mellitus is more in males. This is a conflicting result of risk calculation. The conflicts arose out of confounding of insulin secretion by β cell dysfunction.

11.5.5. Partitioning of fasting insulin and the proposal for disease predisposition and risk calculation with reference to hyperinsulinemia

The prevalence of type 2 diabetes mellitus is increased in offspring with FH (Valdez et al, 2007). There are many components reported to be contributing to hyperinsulinemia. They are insulin resistance, increased insulin secretion (Khan, 2003; Gerich, 2003; Weyer et al, 1999), hepatic uptake of circulating insulin by insulin receptors, regulation of insulin degradation (Pivovarova et al, 2013; Mittelman et al, 2000; Ferrannini and Balkau, 2002), premenopausal women with increased insulin sensitivity (Jarvis et al, 2013; Park et al., 2003; Yki-Jarvinen 1984) and FH (Haffner et al, 1988). In this study fasting insulin was partitioned according to FH and according to gender (Table 11.7). Fasting insulin increases from group I to group II and from group II to group IIIa and then to group IIIb (chapter 7). There is increased fasting insulin in males of group II and III (chapter 8). These criteria were used to partition fasting insulin as given in table 11.7.

Hyperinsulinemia is related to family history of type 2 diabetes mellitus (Haffner et al.1988). FH increases the prevalence of type 2 diabetes mellitus in offspring. Therefore, hyperinsulinemia may be related to increased risk of type 2 diabetes mellitus. Also, FH will result in increased predisposition of type 2 diabetes mellitus in offspring. There is increased hyperinsulinemia with male gender. Therefore male gender has increased predisposition to type 2 diabetes mellitus (Table 11.8).

When the level of fasting insulin is above the 95% reference interval of a particular group, that level of insulin shows a higher risk of type 2 diabetes

mellitus than those individuals within the reference interval of particular group (Table 11.8).

Risk calculation was done by odds ratio (Table 11.8). Group I, the control group has odds ratio equal to 1. The odds ratio increases from group I to group II and from group II to III, in male and females, when there is a common comparable cutoff level (Table 11.3). When there is a common comparable cutoff level for males and females the odds ratio for group II was higher in males, but was almost same for males and females in group III (Table 11.3).

To conclude there is an increased predisposition and increased risk of type 2 diabetes mellitus with FH in male participants

11.5.6. Partitioning of fasting C peptide and the proposal for disease predisposition and risk calculation with reference to hyperinsulinemia

There was no influence of family history of type 2 diabetes mellitus for fasting C peptide. There was a gender difference with increased fasting C peptide in males in group II and III (Table 11.9). There was no gender difference for fasting C peptide in group I.

When the fasting C peptide levels were above 95% reference interval in a particular group there was increased predisposition to type 2 diabetes mellitus than when the fasting C peptide was within the reference interval of that particular group (Table 11.10).

The risk of increased insulin secretion is calculated by odds ratio for fasting C peptide with common cutoff (Table 11.6). Fasting C peptide increased from group I to group II in males and females indicating increased insulin secretion in group II. But in group III fasting C peptide decreased from the level in group II indicating confounding of increased insulin secretion by β cell dysfunction. This phenomenon happened in both males and females samples of fasting C peptide.

The male odds ratio of fasting C peptide was higher in group II. But the males and females fasting C peptide odds ratio in group III cannot be compared as both values were lower, probably due to confounding. To conclude fasting C peptide may be grouped in to FH groups I, II and III. The FH groups II and III may be sub partitioned according to gender.

Table 11.1. Fasting insulin range, 95% confidence interval of mean and 95% reference interval of various groups partitioned according to family history of type 2 diabetes mellitus and gender.

Fasting Insulin	**Range**	**95% CI of mean**	**95% Reference Interval (pmol/L)**
Group I n = 23	8.46 – 37.32	18.79 – 25.22	8.5 - 37.3
Group II male n = 22, Box - Cox λ = (-0.23)	19.86 – 80.94	32.97 – 46.63	19.9 - 80.9
Group II Female n = 38	13.68 – 55.32	29.61 – 35.89	12.8 - 52.2
Group III male n = 19, Box-Cox λ = (-0.7)	20.88 – 106.14	36.02 – 60.45	20.9 - 106.1
Group III female n = 34, Box-Cox λ = (0.35)	12.24 – 97.56	36.17 – 49.41	12.8 - 90.9

Table 11.2. Calculation of cutoff values for determination of odds ratio in male and female samples with reference to fasting insulin.

	Calculation for cutoff value	
Male	Average of upper limit of 95% CI of mean of group I and lower limit of 95% CI of mean of group II male	$\frac{25.22 + 32.9}{2} = 29.1$
Female	Average of upper limit of 95% CI of mean of group I and lower limit of 95% CI of mean of group II female	$\frac{25.22 + 29.61}{2} = 27.4$

Table 11.3. Calculation of odds and odds ratio of various partitioned groups for fasting hyperinsulinemia

Cut-off values of Fasting Insulin	Groups	Above/Below	Odds	Odds Ratio	Relative Risk	Percentage
Male 29.1	Group I	3/20	0.15	1	0.130	13.0
	Group II	18/4	4.5	30	0.818	81.8
	Group III	15/4	3.75	25	0.789	78.9
Female 27.4	Group I	5/ 18	0.28	1	0.217	21.7
	Group II	26 / 12	2.17	7.8	0.684	68.4
	Group III	29/5	5.8	20.88	0.853	85.3
Common cutoff 27.4	Group II male	18/4	4.5	16.07	0.818	81.8
	Group III male	16/3	5.33	19.04	0.842	84.2

Table11.4. Fasting C peptide range, 95% confidence interval of mean and 95% reference interval of various groups partitioned according to family history of type 2 diabetes mellitus and gender

Fasting C peptide	Range	95% CI of mean	95% Reference interval (nmol/L)
Group I **n = 16**	0.35 – 0.78	0.45 – 0.59	0.3 – 1.2
Group II male **n = 11** **Box-Cox λ = (-0.24)**	0.49 – 1.02	0.58 – 0.82	0.5 – 1.02
Group II female **(n = 24)** **Box-Cox λ = 1.21**	0.33 – 0.79	0.51 – 0.64	0.3 – 0.8
Group III male **n = 11** **Box-Cox λ = (-1.31)**	0.33 – 1.19	0.56 – 0.96	0.3 – 1.2
Group III female **n = 18** **Box-Cox λ = (-0.21)**	0.36 – 1.13	0.46 – 0.60	0.4 – 1.1

Table11.5. Calculation of cutoff values for determination of odds ratio in male and female samples with reference to fasting C peptide.

	Calculation for cutoff value	
Male	Average of upper limit of 95% CI of mean of groupI and lower limit of 95% CI of mean of groupII male	$\frac{0.59 + 0.58}{2} = 0.585$
Female	Average of upper limit of 95% CI of mean of groupI and lower limit of 95% CI of mean of groupII female	$\frac{0.59 + 0.51}{2} = 0.55$

Table11.6. Calculation of odds and odds ratio of various partitioned groups for fasting hyperinsulinemia

Cut-off values of fasting C peptide	Groups	Above / Below	Odds	Odds Ratio	Relative Risk	Percentage
Male 0.585	Group I	3/13	0.23	1	0.188	18.8
	Group II	8/3	2.67	11.56	0.727	72.7
	Group III	7/4	1.75	7.58	0.636	63.6
Female 0.55	Group I	6/10	0.6	1	0.375	37.5
	Group II	15/9	1.67	2.78	0.625	62.5
	Group III	9/9	1	1.67	0.5	50.0
Common cutoff 0.55	Group II male	9/2	4.5	7.5	0.818	81.8
	Group III male	4/7	0.57	0.95	0.363	36.3

Table 11.7. Proposal for partitioning fasting insulin into groups and subgroups. Fasting insulin levels in subgroups of group II were not different and sub partitioning was not done. There were no gender differences of fasting insulin in group I.

Partitioning the familiality of type 2 diabetes mellitus into Groups and Subgroups	**n (in males and females)**	**Criteria of partitioning familiality of type 2 diabetes**
I	23	Parents and grandparents are not diabetic. No gender difference in fasting insulin.
II **[II(m) + II(f)]**	60 (22 + 38)	Parents are not diabetic but one or more of grandparents are diabetic. Could be sub partitioned into male and female subgroups.
III **(IIIa + IIIb)** **[III(m) + III(f)]**	53 (41 + 12) (19 + 34)	Any one (IIIa) or both (IIIb) parents are diabetic. Sub partitioned into male (m) and female (f) subgroups.
(II + III) **[(II+III)(m) + (II+III)(f)]**	113 (41 + 72)	Participants with family history of diabetes in parents or grandparents. Sub partitioned into male and female subgroups.
Total Sample **(I+II+III)**	136	All participants in the study.
Noteon Group II: If in a participant, FH in grandparents could not be established and parents do not have diabetes but siblings of parents have diabetes, then the participant was then included in Groups IIa for fasting insulin calculations.		

Table 11.8. Genetic and gender difference in the predisposition to hyperinsulinemia (type 2 diabetes mellitus) and the calculation of risk of hyperinsulinemia by odds ratio.

Familiality Groups	**Genetic predisposition to type 2 diabetes (Hyperinsulinemia)**	**Within 95% reference interval of fasting insulin**	**Above 95% reference interval of fasting insulin**	**Gender difference in type 2 diabetes predisposition**	**Risk of hyperinsulinemia by odds ratio**
I	Low	8.5 – 37.3		No gender difference	1
II	Intermediate	Male : 19.9 – 80.9 Female: 12.8 - 52.2	Male >80.9 Female>52.2	Increased predisposition in male	Male:16.07 Female:7.8
III a	High	Male : 20.9 -106.1 Female: 12.8 - 90.9	Male >106.1 Female>90.9	Increased predisposition in male	Male: 19.04 Female: 20.88
III b	Very high			Increased predisposition in male	

Table 11.9. Proposal for partitioning of fasting C peptide into groups. Fasting C peptide groups I, II and III were not different from each other. There were no influence of FH on fasting C peptide.

Partitioning the familiality of type 2 diabetes mellitus into Groups and Subgroups	**n (in males and females)**	**Criteria of partitioning familiality of type 2 diabetes**
I	16	Parents and grandparents are not diabetic. There was no gender difference, so no partition for gender
II **[II(m) + II(f)]**	35 (11 + 24)	Fasting C peptide is higher in group II males than females
III **[III(m) + III(f)]**	29 (11 + 18)	Fasting C peptide is higher in group III males than females
(II + III) **[(II+III)(m) + (II+III)(f)]**	64 (22 + 42)	There was gender difference in fasting C peptide in individuals with FH
Total Sample **(I+II+III)**	80	Sample population reference interval

Table 11.10. Gender differences in the predisposition to increased fasting C peptide (type 2 diabetes mellitus) and the calculation of risk increased insulin secretion by odds ratio.

Familialit y Groups	**Genetic predispositi on to type 2 diabetes (Increased insulin secretion)**	**Within 95% reference interval of fasting C peptide**	**Above 95% reference interval of fasting C peptide**	**Gender difference in type 2 diabetes predispositio n**	**Risk of hyperinsuli nemia by odds ratio**
I	Low	0.3 - 1.2	>1.2	No gender difference	1
IImale	Higher in males	0.5 - 1.02	>1.02	Increased predisposition in male	7.5
IIfemale		0.3 - 0.8	> 0.8		2.78
IIImale	Higher in females	0.3 - 1.2	>1.2	Increased predisposition in male	0.95
IIIfemale		0.4 - 1.1	> 1.1		1.67

SUMMARY AND CONCLUSION

Reference values are required for all clinical laboratory tests, not only from healthy individuals but also from patients with a clinically suspected disease.Some conditions are required to make the comparison of patient's laboratory results with reference values possible and valid (IFCC 1987-1991; Mardia 1980; Solberg & Grasbeck, 1989).The population from which reference data is collected should be defined. All the groups of reference individuals should be clearly defined with inclusion and exclusion criteria. Conditions in which the sample were obtained and processed for analysis should be known.All quantities compared should be of same type.All laboratory results should be produced with the use of adequately standardized methods under sufficient analytical quality control.

The reference interval calculated in this study may be defined as the baseline reference interval. They were calculated for insulin and C peptide with reference to type 2 diabetes mellitus. The population defined for the baseline reference interval may be without the influence of growth phase (excluding participants below 18 years), without significant environmental influences (excluding individuals above 25 years) and healthy participants without secondary clinical or subclinical influences. The subclinical disease states were identified by clinical biochemistry laboratory asssays.

Partitioned groups are better representatives of baseline reference interval. It was shown that family history of type 2 diabetes mellitus up to grandparents and gender of the participants of the study influence reference interval of fasting insulin and C peptide. Therefore, partitioned reference interval can be more useful for clinical interpretation of fasting insulin and C peptide levels.

In chapter 3, the relationship of fasting insulin with post glucose load OGTT insulin was analysed in young adults. This was followed by partitioning fasting and post glucose load OGTT serum insulin according to family history of type 2 diabetes. Hyperinsulinemia is related to family history of type 2 diabetes mellitus. Family history of type 2 diabetes mellitus indicates genetic predisposition to type 2 diabetes mellitus. Fasting insulin correlated well with all post glucose load insulin of OGTT. Fasting, 60 and 120 minute OGTT insulin can be partitioned according to family history of type 2 diabetes mellitus. Coefficient of variation was

least for fasting insulin. Therefore, fasting insulin was chosen for detailed analysis of the influence of family history of type 2 diabetes and gender differences.

In chapter 4, correlation of fasting C peptide with fasting insulin and with OGTT C peptide in young adults were analyzed. Partitioning of fasting C peptide and OGTT C peptide was according to family history of type 2 diabetes in parents.C peptide represents insulin secretion. Increase in insulin secretion is seen as a compensation for insulin resistance. Fasting C peptide correlates with fasting insulin and with OGTT C peptide in young adults but unlike insulin, C peptide groups could not be partitioned according to family history of type 2 diabetes mellitus. Therefore, fasting C peptide may be considered for analysis of the influence of gender on insulin secretion.

In chapter 5, the correlation between fasting C peptide with oral glucose tolerance test (OGTT) C peptide, and the gender differences in the mean C peptide after partitioning were analysed. All the three parameters related to insulin resistance, fasting insulin, triglycerides and fasting C peptide were increased in the male sample with family history of type 2 diabetes. These were required for understanding the future risk of hyperinsulinemia and in the clinical interpretation of fasting C peptide levels. Significant partitioning could be done in fasting C peptide in the group with family history of type 2 diabetes in males and females.

In chapter 6 we evaluated the gender difference in the correlation of fasting insulin with OGTT insulin and related parameters, and in the gender difference in the mean fasting insulin in various groups were analyzed. There was gender difference in the correlation of fasting insulin with OGTT insulin groups. This difference was found to be due to the strong correlation seen in female samples with family history of type 2 diabetes mellitus. The mean fasting insulin and fasting triglyceride were higher in the male samples and in the samples with family history of type 2 diabetes mellitus. It was concluded that there are gender differences in the fasting insulin levels in groups with family history of type2 diabetes mellitus. The gender differences may be attributed to increased insulin sensitivity in premenopausal women and increased beta cell dysfunction in men with family history of type 2 diabetes mellitus.

In chapter 7, evaluation of the influence of family history of type 2 diabetes mellitus upto grandparents on fasting insulin in young adults aged 18 to 25 years was done.Participants were partitioned into familiality groups I (no FH), II (FH in grandparents, not in parents) and III (FH in parents), and sub partitioned into subgroup IIIa (one parent diabetic) and IIIb (both parents diabetic). Mean fasting insulin increased from group I to II, from II to III and from subgroup IIIa to IIIb. But subgroups of group II, partitioned according to the number of grandparents with diabetes, had similar fasting insulin levels. This study showed that it is possible to establish the baseline fasting insulin reference intervals in each familiality group and sub group.

In chapter 8, we analysed the influences of gender and family history of type 2 diabetes mellitus on fasting insulin in young participants before and after partitioning the sample according to gender and family history of type 2 diabetes mellitus. One way ANOVA of fasting insulin showed that the three familiality groups were different in males ($P = 0.001$) and in females ($P = <0.001$), and males had consistently higher mean insulin than females in groups II ($P = 0.051$) and III ($P = 0.413$). Fasting insulin in females from groups II and III were normally distributed, but not in males. Gender differences in FH and its influence on fasting insulin are required for understanding future risk of hyperinsulinemia.

In chapter 9, analysis of the influence of family history of type 2 diabetes mellitus upto grandparents on fasting C peptide and their gender differences were done. It was found that there is least influence of FH on fasting C peptide, but the gender difference in groups with FH was highly significant.

In chapter 10, we calculated the 95% reference intervals of the total and partitioned data of fasting insulin and fasting C peptide and the results were as follows:

The **95% reference intervals for fasting insulin** : Total fasting insulin: 12.6 - 92.9 pmol/L (non parametric method); Group I (male and female): 8.5 - 37.3 pmol/L (non parametric range); Group II male 19.9 - 80.9 pmol/L (non parametric range); Group II female: 12.8 - 52.2 pmol/L (parametric); Group III male: 20.9 - 106.1 pmol/L (non parametric range); Group III female: 12.8 - 90.9 pmol/L (parametric).

The **95% reference intervals for fasting C peptide**: Total fasting C peptide: 0.3 – 1.1 nmol/L (parametric method); Group I (male and female): 0.3 – 1.2 nmol/L (non parametric range); Group II male 0.5 – 1.02 nmol/L (non parametric range); Group II female: 0.3 – 0.8 nmol/L (non parametric range); Group III male: 0.3 – 1.2 nmol/L (non parametric range); Group III female: 0.4 – 1.1 nmol/L (non parametric range).

In chapter 11, we analysed the predisposition to type 2 diabetes and the risk of hyperinsulinemia and increased insulin secretion by odds ratio. Insulin secretion was represented by fasting C peptide in males and females.In this study, the risk of hyperinsulinemia was calculated by identifying a cutoff level of fasting insulin between the control group I and group II. The midpoint between the 95% CI of mean of fasting insulin of these groups was taken as the cutoff value. With this cutoff value the odds were calculated for groups I, II and III. The odds ratio was estimated for groups II and III with reference to group I for calculating the risk of hyperinsulinemia. This risk increased from group I to II and from group II to III for both males and females. The risk was also higher in males.

In the case of fasting C peptide, the levels were increased in males, indicating increased insulin secretion in male sample. Insulin secretion increased with increased insulin resistance. The odds ratio was higher in group II and III, but group III had lower value than II. But odds ratio of group III male and female samples were not dependable as they showed severe confounding of insulin secretion by β cell dysfunction. Odds ratio for the male samples were higher than that of the female sample, indicating higher insulin resistance and higher risk of type 2 diabetes in males.

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