UNIVERSITY OF LIVERPOOL

INVESTIGATING THE INCRETIN SYSTEM IN SOUTH ASIANS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree M.D.

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3rd March 2020

UNIVERSITY OF LIVERPOOL

Declaration

The study design presented in this thesis is a collaborative effort of me and my supervisors Professor Jiten Vora and Professor L Ranganath.

In addition, I have also received guidance from Professors Tina Vilsboll and Filip Knop (Gentofte group, Copenhagen) as well as Professor Juris Meier (Germany).

I secured a Society of Endocrinology lab grant (2011) to visit Professor T Vilsboll's and Professor Filip Knop's group at Gentofte Hospital, Copenhagen to gain experience in performing isoglycaemic clamp studies.

In addition, I also visited Professor Jens Holst's laboratory at the Panum Institute, Copenhagen following successful funding as part of the Samuel Leonard Simpson Fellowship grant by the Royal College of Physicians (2012). My visit to the Panum Institute was aimed at gaining laboratory experience of incretin assays as well as incretin studies performed in animals including pigs.

For my research study, I recruited all subjects, performed experimental work including the clamp studies, collected blood samples, analysed and presented the data in this thesis. The laboratory analyses of blood samples were carried out at

accredited laboratories at the Royal Liverpool and Broadgreen University hospitals NHS Trust and the Panum Institute (Professor Jens Holst's laboratory), Copenhagen. A small part of the preliminary experimental analysis (comparing NEFA responses post liquid meals) was done by our Master of Research (MRes) student, Muhammad Khan as part his MRes Clinical Sciences (project 3; 200652269, University of Liverpool)

Statistical support was provided by Dan Lythgoe, statistician, University of Liverpool.

Glossary

ADA. American Diabetes Association

BMI. Body Mass Index

CHO. Carbohydrate

DI. Disposition Index

DPP4. Dipeptidyl Peptidase-IV

FPG. Fasting plasma glucose

GIP. Gastric Inhibitory Peptide

GLP-1. Glucagon like peptide -1

HbA1c. Glycated Haemoglobin

IDDM. Insulin Dependent Diabetes Mellitus

IFCC. International Federation of Clinical Chemistry

IFG. Impaired Fasting Glucose

IGT. Impaired Glucose Tolerance

ISR. Insulin Secretion Rate

NEFA. Non-esterified fatty acids

NGT. Normal Glucose Tolerance

NIDDM. Non-Insulin Dependent Diabetes Mellitus

OGTT. Oral Glucose Tolerance Test

PYY. Peptide Tyrosine Tyrosine

T1DM. Type 1 Diabetes Mellitus

T2DM. Type 2 Diabetes Mellitus

WHO. World Health Organisation

ABSTRACT

South Asians are known to have an increased risk of type 2 diabetes, at lower levels of adiposity and at a younger age, in comparison to Caucasians. In addition, there is evidence of insulin resistance amongst South Asians, from early years of life. The exact mechanisms for this increased risk are not fully understood.

Incretins are insulinotropic intestinal hormones accounting for a significant amount of postprandial insulin release. Both GLP-1 and GIP are now recognised as key hormones responsible for the incretin effect. The incretin effect is impaired in type 2 diabetes and remains a pivotal therapeutic target with the advent of incretin-based drugs. There is emerging evidence that incretin hormones may vary amongst Asians. Incretin-based drugs have been found to be more efficacious amongst Asians including South Asians. However, there are very limited mechanistic incretin studies amongst South Asians.

We compared two key aspects of the incretin hormone responses between normal glucose tolerant South Asians and Caucasians.
This included comparing the incretin effect as well as total GLP-1 and GIP responses to isocaloric liquid meals with varying compositions (carbohydrate rich, mixed and fat rich meals).
Despite the presence of hyperinsulinaemia, normal glucose tolerant South Asians had a comparable incretin effect following a 50-gram glucose load.

To the best of our knowledge, this is the first study making contemporaneous comparisons between incretin hormone responses between normal glucose tolerant South Asians and Caucasians.

Acknowledgments

My research would not have been possible without the support of many people.

I am greatly indebted to Professor Jiten Vora for his constant encouragement, guidance, support and inspiration. He has been an extremely knowledgeable, and an inspiring mentor to me. He has helped me to evolve both as a clinician as well as a researcher.

I am grateful to Professor L Ranganath for his supervision and constant guidance throughout my research. His immense experience in well cited incretin studies provided insightful direction for the study.

I am also incredibly grateful to Sister Helen Bygott for assisting me with sampling during many very tedious and time pressured experiments.

Also, very grateful to Bill Taylor, June Devine and Jeanette Usher for their relentless laboratory support.

I am extremely grateful to Professors Tina Vilsboll and Filip Knop for their collaboration and incredible support at various stages of the study and beyond. They helped me to acquire skills in performing isoglycaemic clamp studies. I am also grateful to them for providing me with a DPP-4 inhibitor powder required for sampling.

I am also grateful to the Royal College of Physicians for the Samuel Leonard Fellowship grant as well as the Society of Endocrinology to enable me to visit the Panum Institute (Professor Jen Holst's laboratory) as well the Gentofte group (Professors Tina Vilsboll and Filip Knop). My research would also not be complete without the extraordinary support of Professor Jens Holst and his group who analysed my samples for GLP-1, GIP and glucagon.

I am also very grateful to Professor Juris Meier for his guidance, support and input especially in designing the study.

Professor Dan Cuthbertson (Aintree University Hospital, Liverpool) loaned me the YSI glucose analyser for which I am very grateful.

Also thankful to Professor Roman Hovorka (Cambridge) for assisting me in calculating the insulin secretion rates using the ISEC programme and Professor Masafusmi Matsuda (Japan) for his assistance in using the Matsuda index calculator.

Also, very thankful to Heather Jones (R&D manager, RLBUHT) and the Clinical Research Facility team who helped me to facilitate the study.

Lastly, I am extremely grateful to all who volunteered to participate in my study without whom this work would have never been possible.

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Introduction

1.1 History

Diabetes was first recognized around 1500 B.C. when Egyptians considered it a condition resulting in excessive urination and weight loss. The term diabetes mellitus was used to suggest that the urine tasted sweet and was first used by the Greek physician Areteaus. The term Diabetes is derived from the Greek word for 'Siphon' and 'Mellitus' or sweet was a subsequent addition to the term to distinguish it from another polyuric condition 'Diabetes Insipidus', in which urine was tasteless. However, it was not until 1776 when Matthew Dobson measured glucose in the urine of these patients and found it to be raised (Dobson 1776).

Subsequently the first link between the pancreas and glucose regulation was made much later by Oskar Minkowski and Josef von Mering. In 1889 the pair alluded to this connection when they found that the removal of pancreas from dogs led to fatal diabetes.

The discovery of islet cells by Paul Langerhans in 1869 was a landmark event in the history of diabetes. It was in 1910 when the hypothesis of diabetes resulting due to deficiency of a chemical produced from the pancreas, was proposed by Sir Edward Albert Sharpey-Schafer. He named the chemical insulin after the Latin word insula referring to the islets of Langerhans in the pancreas.

Subsequently the most significant development was the discovery of insulin by Banting and Best in 1921. Banting and Best discovered insulin after they reversed diabetes that had been induced in dogs, with an extract from the pancreatic islets of healthy dogs (1).

James Collip and John Macleod purified the hormone insulin from bovine pancreases and were the first to use it to treat a patient with diabetes. Eventually Frederick Sanger in 1945 identified the insulin amino acid sequence leading to a revolution in the treatment of diabetes (2). Sanger was awarded the Nobel prize in Chemistry, for his

work relating to insulin. Insulin was also the first hormone to be cloned and then produced for therapeutic use by means of recombinant DNA technology (3).

Rosalyn Yalow and Solomon Berson developed the first radioimmunoassay for insulin in 1959, leading to transformation of the future of diabetes (4).

Much of our understanding in diabetes has resulted due to our ability to measure insulin levels.

However, given the recent increasing prevalence of diabetes, earlier diagnosis, evolving understanding of its pathogenesis with an expanding armamentarium of available treatment options, diabetes still remains a challenging condition on various fronts.

1.2 Global prevalence

The prevalence of diabetes continues to increase at an alarming rate around the world making it one of the most challenging health problems in the 21st century. The estimated worldwide prevalence of diabetes has risen from 285 million in 2010 to 425 million as per the latest International Diabetes Federation statistics (5). This number to expected to rise to 629 million by 2045. Type 2 diabetes is the predominant form of diabetes accounting for at least 90% of cases (5).

1.3 Diagnosis & classification of diabetes

The diagnosis and classification of diabetes has remained a topic of discussion for a very long time. The diagnostic criteria for diabetes have changed over the years.
Since 1965 the World Health Organisation (WHO) has published guidance and criteria for the diagnosis and classification of diabetes(6). A fasting blood sugar (glucose) value ≥7.2 mmol/L was the recommended criteria for diagnosis (6). Subsequent revisions were made over the next three decades.
In 1979, the National Diabetes Data Group, proposed new criteria and introduced the terms 'Non-Insulin Dependent Diabetes Mellitus' (NIDDM) and 'Insulin Dependent Diabetes Mellitus' (IDDM) to distinguish the two main types of diabetes and also included the term 'Impaired Glucose Tolerance' (IGT) (7). The American Diabetes Association (ADA), in 1997, revised the criteria for diagnosis to a new lower threshold for fasting blood glucose of 7 mmol/L and introduced a new category 'Impaired Fasting Glucose' (IFG) for those with fasting glucose values between 6 and 6.9 mmol/L(8).

There are small differences between the WHO and ADA criteria(9). While the ADA consider a cut-off value of 5.6 mmol/L for IFG based on studies on Pima Indians and other ethnic groups (10), the WHO cut-off value remains at ≥6.1 mmol/L. The WHO recommendations are based upon diagnostic criteria being able to distinguish a group with significantly increased premature mortality and increased risk of microvascular and cardiovascular complications.

1.4 Diagnostic tests for diabetes

Historical perspective

The concept of an oral glucose tolerance test was simultaneously introduced by Hamman and Hirschman (1917) in the US and by Jacobson (1913) in Denmark. Further to that, an unsuccessful attempt to introduce an intravenous glucose tolerance test to diagnose diabetes was also made in 1923 (Jorgensen 1923). The development of insulin radioimmunoassay back in the 1960s led to the identification that people with

'early maturity onset diabetes' produced insulin and secreted this hormone in response to nutrient ingestion (11).
Subsequently, a 2-hr. OGTT remained one of the main diagnostic modalities till 1979. However, these were based on studies on Pima Indians who demonstrated a bimodal distribution while in general population it was unimodal. In 1979, the first ever, consensus classification system was introduced by the national diabetes data group. These recommendations were further endorsed by WHO in 1980 (12).

Over the years, the criteria for diagnosis of diabetes have changed. Unlike previous increased reliance on the oral tolerance tests, diagnosis in clinical practice is now increasingly made with single tests such as glycosylated haemoglobin.

Oral Glucose Tolerance Test (OGTT):

There is considerable debate about the role of the OGTT in the diagnosis and classification of diabetes. A number of studies have reported that fasting plasma glucose and 2-hour post-glucose plasma glucose do not identify the same people as having diabetes. Using only fasting plasma glucose criteria will fail to diagnose approximately 30% of people with diabetes. There are documented increased rates of mortality and worse outcomes in relation to diabetes diagnosed on the basis of the 2-hour plasma glucose result. The Hoorn study showed that all cause cardiovascular mortality over a 8 year follow-up was significantly elevated in those with a 2 hour plasma glucose ≥11.1 mmol/L but not in those with a fasting plasma glucose ≥7 mmol/L (13). The 2-hour plasma glucose is also important for microvascular complications with an increased incidence of retinopathy in cases newly diagnosed diabetes with 2-hour values ≥11.1 mmol/L and even in those with a fasting value ≥7 mmol/L.

Hence WHO recommended that the OGTT is an important test and is the only means of identifying people with IGT and is an important exclusion test in asymptomatic people. The WHO recommends that the OGTT be used, with fasting glucose values between 6.1 and 6.9 mmol/L to determine glucose tolerance status (12).

The test is recommended by the WHO and although the ADA acknowledges the OGTT as a valid diagnostic test, in clinical practice it is inconvenient, costly and poorly reproducible.

Glycated Haemoglobin (HbA1c):

HbA1c was identified in 1969 as an unusual haemoglobin in patients with diabetes and subsequently numerous studies correlating HbA1c to glucose measurements suggested it could be used as an objective measure of glycaemic control.
Since the 1980s HbA1c has been widely used in clinical practice and reflects average plasma glucose over the previous 8 – 12 weeks. It soon became the preferred test for assessing glycaemic control in diabetics as it could be performed any time in the day and did not require any special preparation or fasting (14). In addition, the HbA1c measure bypassed the problem of variability of daily glucose levels. The WHO, after initially rejecting the test, revised its guidance to include this as a diagnostic test. An HbA1c value of 6.5% (48 mmol/mol IFCC) is recommended as the cut off point for diagnosing diabetes (WHO 2011).

Use of HbA1c has advantages over tests like fasting glucose and oral glucose tolerance test, including convenience (fasting not needed), greater pre analytical stability and less day-to-day perturbations particularly during stress and illness.
However, there are limitations in using HbA1C including lower sensitivity at designated cut off point, greater cost, limited availability of HbA1c testing in certain parts of the world and lack of concordance with HbA1c and average glucose in certain individuals (15).

Data from the National Health and Nutrition Examination Survey (NHANES) indicates that a HbA1C cut off of ≥ 6.5% (48 mol/mol) identifies a prevalence of undiagnosed diabetes that is only one third of that using the glucose criteria (16).
While using HbA1c, we also need to remain mindful of factors that may affect haemoglobin glycation independent of glycaemia including age, race/ethnicity and anaemia/haemoglobinopathies (15).

Use of HbA1c as a diagnostic modality was based on evidence from epidemiological studies including only adult population. Therefore it remains unclear if HbA1c can still be used as a valid diagnostic test for paediatric and adolescent populations (17).

Significant discrepancy between glucose levels and measured HbA1c in an individual should prompt further review.

While most common haemoglobin variants do not interfere with the HbA1c assays, there are some such as sick cell trait which require blood samples to be analysed on HbA1c assay without interference, for greater accuracy
(National Glycohemoglobin Standardization Program. NGSP: HbA1c assay interferences. http://www.ngsp.org/interf.asp).

There are also some rare forms of haemoglobin variants found amongst African Americans that can result in interference causing discordant HbA1c results (18, 19).

In addition, even in the absence of haemoglobin variants, individuals of certain ethnicity have variation in HbA1c independent of glycaemia, suggestive of racial differences (20, 21). In particular African Americans appear to have higher HbA1c compared to non-Hispanic whites despite similar fasting and postprandial glucose load (22) and HbA1c levels may be higher for a given mean glucose level when measured with continuous glucose monitoring (23). There is some evidence to suggest that African Americans may have increased levels of fructosamine and glycated albumin and lower levels of 1,5- anhydroglucitol suggesting that there glycaemic burden may be higher.
Interestingly the association of HbA1c with risk of complications appears to be similar for African Americans and non-Hispanic Whites (24, 25).

Red blood cell turnover:
Conditions such as pregnancy, sickle cell disease, haemodialysis, blood loss or transfusion, are associated with increased red blood cell turnover make HbA1c unreliable (26).

1.5 Diagnostic criteria for diabetes

Table 0.1 ADA criteria for the diagnosis of diabetes (15)

FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h PG ≥200 mg/dL (11.1 mmol/L) during OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water. *
OR
HbA1c ≥6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.
OR
In a patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).

* In the absence of unequivocal hyperglycaemia, results should be confirmed by repeat testing.

Table 0.2 WHO criteria for diagnosis of diabetes(12)

Diabetes	
Fasting plasma glucose	≥7.0mmol/l (126mg/dl)
2–h plasma glucose*	**or** ≥11.1mmol/l (200mg/dl)
Impaired Glucose Tolerance (IGT)	
Fasting plasma glucose	<7.0mmol/l (126mg/dl)
2–h plasma glucose*	**and** ≥7.8 and <11.1mmol/l (140mg/dl and 200mg/dl)
Impaired Fasting Glucose (IFG)	
Fasting plasma glucose	6.1 to 6.9mmol/l (110mg/dl to 125mg/dl)
2–h plasma glucose*	**and (if measured)** <7.8mmol/l (140mg/dl)

* Venous plasma glucose 2–h after ingestion of 75g oral glucose load

* If 2–h plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded

1.6 Classification of diabetes

The first classification of diabetes was made by WHO in 1980(12) while the 75 g oral glucose tolerance test(OGTT) became the gold standard with defined fasting and 2-hour values . This has then been modified over the last three decades. Since 1998, a new classification based on collaboration between the WHO and ADA groups has been in use (8, 27).

However, it's now widely accepted that there are broadly three main types, namely type 1, type 2 and gestational diabetes.

Broadly diabetes can be classified into five different categories as follows (15):

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

2. Type 2 diabetes (due to a progressive loss of β-cell insulin secretion frequently on the background of insulin resistance)

3. Gestational diabetes mellitus (GDM) (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation).

4. Monogenic - Rare types of diabetes due to other causes, e.g., monogenic diabetes syndromes (such as neonatal diabetes and maturity-onset diabetes of the young [MODY]).

5. Other- diseases of the exocrine pancreas (such as cystic fibrosis and pancreatitis), and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS or after organ transplantation).

Type 1 diabetes is now also further categorized into three stages based on disease progression (28, 29)(table 1.3).

This is significant shift from our previous understanding of the pathogenesis of type 1 diabetes. It's now known that the rate of beta cell destructions in type 1 diabetes is not absolutely linear, rather more typical of relapsing and remitting diseases (30).
Data from the TEDDY trial showed that the autoantibodies' against pancreatic islets may occur much earlier in life and well before the onset of over diabetes for some individuals alluding to an opportunity for immune interventions (31).
Given limited success of recent secondary prevention trials including stem cell transplant aimed at immunological reset, altering the natural history of type 1 diabetes remains a focus of intense ongoing research (32).

Table 0.3: Staging of type 1 diabetes (15)

	Stage 1	Stage 2	Stage 3
Characteristics	• Autoimmunity	• Autoimmunity	• New onset hyperglycaemia
	• Normoglycemia	• Dysglycaemia	• Symptomatic
	• Presymptomatic	• Presymptomatic	
Diagnostic criteria	• Multiple autoantibodies	• Multiple autoantibodies	• Clinical symptoms
	• No IGT or IFG	• Dysglycaemia: IFG and/or IGT	• Diabetes by standard criteria
		• FPG 100–125 mg/dL (5.6–6.9 mmol/L)	
		• 2-h PG 140–199 mg/dl (7.8–11.0 mmol/L)	
		• A1C 5.7–6.4% (39–47 mmol/mol) or ≥10% increase in A1C	

1.7 Prediabetes

Prediabetes is used to define a state of dysglycaemia where glucose levels are too high to be classed as normal and yet do not meet the diagnostic criteria for diabetes (33). Individuals with prediabetes can be defined by the presence of impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) based on the same parameters used for diagnosis of diabetes. The current diagnostic criteria for prediabetes are as below

Table 0.4: Categories of increased risk for diabetes (prediabetes) (15)

FPG 100 mg/dL (5.6 mmol/L) to 125 mg/dL (6.9 mmol/L) (IFG)
OR
2-h PG during 75-g OGTT 140 mg/dL (7.8 mmol/L) to 199 mg/dL (11.0 mmol/L) (IGT)
OR
HbA1c 5.7–6.4% (39–47 mmol/mol)

↵* For all three tests, risk is continuous, extending below the lower limit of the range and becoming disproportionately greater at the higher end of the range.

WHO diagnostic criteria for prediabetes (WHO 2011)

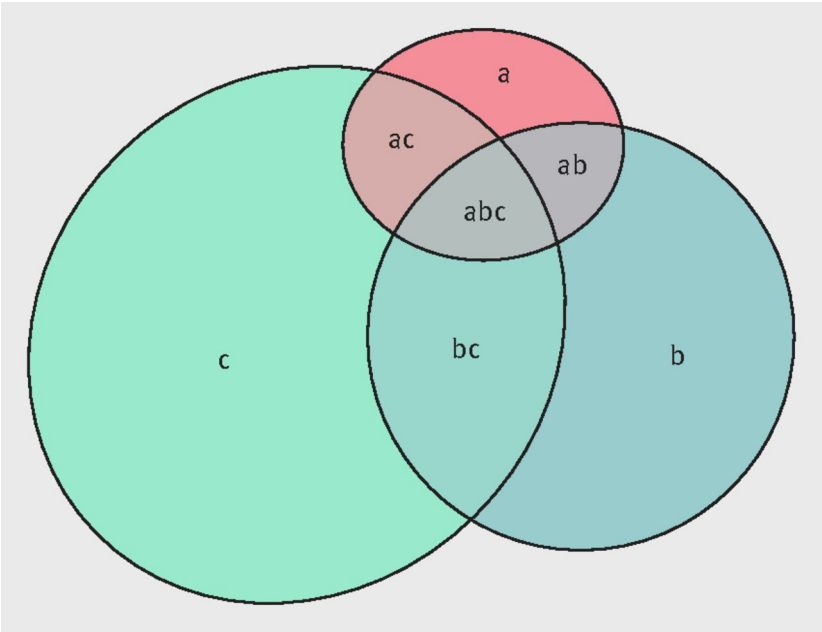
Fasting Plasma Glucose 6-6.9 mmmol/L
Impaired Glucose tolerance
At risk HbA1c 6-6.4%

Individuals with prediabetes are at increased risk of developing type 2 diabetes. The risk is compounded by the presence of obesity, increasing age, positive family history, certain medical conditions such as previous gestational diabetes and polycystic ovarian syndrome and also due to certain ethnicities.
Those with gestational diabetes have a very high risk of progressing to overt type 2 diabetes.

Prediabetes remains a major health and economic challenge across the world.
As per the 8th edition of the IDF atlas, there are 352.1 (233.5–577.3) million people worldwide, who are estimated to have impaired glucose tolerance (IGT) (5). The vast majority (72.3%) of these people live in low and middle-income countries. By 2045, the number of people with IGT is projected to increase to 587 (384.4–992.7) million (5). Nearly one-third (28.8%) of all those who currently have IGT are in the 20-39 age group and are therefore likely to spend many years at high risk and consequently adding to the health-economic burden (5).
However there remains an ongoing debate regarding modalities used for diagnosis of diabetes as well the conundrum of whether as a result of changed criteria and insensitive testing, we are over diagnosing prediabetes (34).

A recent systematic review and metanalysis of screening tests and interventions in prevention of type 2 diabetes, demonstrated that HbA1c is not a sensitive or specific marker for prediabetes (35). The authors reviewed 46 published studies relating to diagnostic accuracy testing. However only five studies (see table) gave a comparison of prevalence of prediabetes for all three tests (HbA1c, fasting plasma glucose, 2 hour glucose tolerance test) (35). Using the various current criteria for prediabetes (IEC, WHO or ADA) the authors noted low agreement between the three tests in people with prediabetes.

Table 0.5 Prevalence analysis of three tests used to identify people with pre-diabetes (Barry BMJ 2017)(35)



Prevalence of prediabetes by diagnostic test with IEC and WHO criteria, showing overlap with all three tests. Prevalence of prediabetes was 27%. Of those with abnormal results, a=4.7% isolated IFG; b=24.4% isolated IGT; c=47.8% isolated HbA1c; ab=2.9% IFG+IGT; ac=4.1% IFG+HbA1c; bc=12.2% IGT+HBA1c; abc=3.9% IGT+IFG+HbA1c; d (area outside ellipse)=72% (normal result)

Diabetes prevention trials have shown that lifestyle interventions are effective particularly in those falling in the category of impaired glucose tolerance.
The three large prediabetes trials were the Diabetes Prevention Programme (36), the Finnish Diabetes prevention study (37)and the Chinese Prevention study (38).

Therefore, prediabetes remains a focus of intense scrutiny and research given the potential to reverse diabetes for individuals detected within this spectrum of disease.

1.8 Type 2 diabetes

Type 2 diabetes (T2DM) is described as a heterogeneous disorder involving insulin resistance, a progressive decline in beta cell function, inappropriate hyperglucagonemia and a reduced incretin effect (39).

Pathophysiology

Following discovery of insulin and its role in glucose regulation, diabetes was viewed as a single hormonal disorder characterized by absolute or relative insulin deficiency.
In the 1950s, glucagon was characterized as a major stimulus of hepatic glucose production. This discovery led to a better understanding of the interplay between insulin and glucagon, thus leading to a bi-hormonal definition of diabetes (40).
A second β-cell hormone, amylin, was first reported in 1987. Amylin was determined to have a role that complemented that of insulin, and, like insulin, was found to be deficient in people with diabetes. This more recent development led to a view of glucose homeostasis involving multiple pancreatic hormones.
In the mid 1970s, several gut hormones were identified, two of which are now well known to contribute to glucose regulation

Type 2 diabetes is now known to result from an interaction between genetic and environmental factors with the genetic background causing insulin resistance and beta cell failure, whereas weight gain and physical inactivity exacerbate these inherited metabolic abnormalities (41).

Insulin resistance involving liver, muscle and adipose tissue is recognised as the core defect in T2DM (41). Insulin resistance develops much earlier in the natural history of T2DM, preceding development of glucose intolerance and overt T2DM (41). Progression of impaired glucose tolerance to T2DM occurs due to a combination of insulin resistance and subsequent beta cell failure (41).

It is now recognised that beta cell dysfunction occurs much earlier in the pathogenesis of T2DM. In addition to the muscle, liver, and beta cell, the fat cell, gastrointestinal

tract, alpha-cell, kidney and brain are also now recognised as important role players in the development of glucose intolerance in T2DM (39).

The 'ominous octet' including muscle, liver, and β-cells, adipocytes (accelerated lipolysis), gastrointestinal tract (incretin deficiency/resistance), α-cells (hyperglucagonaemia), kidney (increased glucose reabsorption), and brain (insulin resistance and neurotransmitter dysregulation) play important roles in development of T2DM (39).

Together these eight players dominate he current therapeutics in T2DM.

The role of gut hormones (incretins) in the pathogenesis of diabetes, is discussed later in the chapter.

1.9 Type 2 diabetes in South Asians

The South Asian population is comprised of individuals originating from India, Pakistan, Sri Lanka, Nepal & Bangladesh. In the United Kingdom, South Asians are the largest ethnic minority group, constituting 4% of the total population.

South Asians are at an increased risk of T2DM with India having the highest global number of patients with diabetes with a predicted prevalence of 79.4 million by 2030 (42).Migrant South Asians have a two to four fold increased risk of type 2 diabetes, the highest risk amongst Bangladeshis and the lowest risk being amongst those of Indian descent.

South Asians develop diabetes earlier in life and at lower levels of adiposity in comparison to Caucasians (42). This has also been shown in the United Kingdom biobank data (43).There is also a predisposition to increased levels of insulin resistance at a lesser degree of obesity amongst South Asians, in comparison to Caucasians (44, 45). As a result there are now recommendations for using ethnically tailored markers of obesity in South Asians (46-49).The annual conversion rate from prediabetes to frank type 2 diabetes is also higher amongst South Asians(50-52). Migrant South Asians are also noted to have more rapidly progressive type 2 diabetes than Europeans (53).

South Asians are also at an increased risk of developing microvascular and macrovascular complications and previously reported to have a higher mortality rate than their White European counterparts (54). Interestingly, a recent UK National Diabetes Audit report showed lower short-term mortality risk in South Asians with type 2 diabetes, in comparison to while Europeans (Health & Social Care Information Centre 2015). This is also in keeping with observations from the study by Shah et al from Canada(discussed below) (55).

The risk of macrovascular complication particularly cardiovascular disease is also increasingly noted to be more comparable to the risk of the background population. This has also been shown in recent studies amongst South Asians living in the UK. This

includes a study from Scotland looking at South Asians diagnosed with type 2 diabetes more recently(56). In this study increased CVD risk was only apparent in Pakistanis and not Indians. In addition there has been a large population based cohort study from Canada looking at South Asians with newly diagnosed type 2 diabetes between 2002 to 2009, noting similar hazard ratios for CHD for South Asians and Europeans (55).

In terms of microvascular complications, South Asians appear to have a higher prevalence of retinopathy, in comparison to White Europeans (57), However the onset of retinopathy doesn't appear to be earlier as suggested by a study whereby South Asians were recruited within 6 months of diagnosis, the incidence of retinopathy was found to be similar to White Europeans (17% vs. 16.6%) (South London Diabetes SOUL-D cohort) (58). This study was aimed at looking at clinical features of South Asians and individuals of Black American or Caribbean ethnicity, at the point of diagnosis.

Therefore, in terms of macrovascular disease, recent change in practice with earlier diagnosis and early multi-pronged therapeutic interventions including use of statins and anti-hypertensives, is likely to drive the overall reduction in cardiovascular mortality amongst South Asians.

At the same time given that the incidence of type 2 diabetes in South Asians is at least a decade earlier with a comparatively higher HbA1c at diagnosis, it would reflect prolonged morbidity with increased retinopathy risk.

Epidemiology (both indigenous and migrant Asians)

South Asians have a much higher risk of type 2 diabetes compared to Caucasians. As per IDF 2017 (5), estimated prevalence of diabetes in South Asia is 4.4% in Nepal and 8.8 % in India. In South Asia, India currently has the largest number of people with diabetes (72.9 million) (5).

The incidence rate of diabetes in South Asians is one of the highest at 22.2 per 1000 person years, next only to Pima Indians (50).

The risk of diabetes amongst South Asians living in the western countries is also higher than the background population.

Increased incidence of type 2 diabetes has been observed even amongst the younger South Asians (59). Overall risk of type 2 diabetes is almost three folds higher amongst migrant South Asians with an almost four-fold higher amongst Bangladeshis compared to almost two-fold amongst Indians.

Irrespective of age group, the incidence of type 2 diabetes remains high amongst South Asians. South Asians are diagnosed with type 2 diabetes almost 5-10 years earlier than the White Europeans at comparatively lower BMI. By the age of 70, almost 30-40% of South Asians have type 2 diabetes, which is twice the prevalence in comparison to White Europeans.

Mechanisms including risk factors

The reasons behind increased risk of type 2 diabetes amongst South Asians are not yet fully understood. Various hypotheses have been proposed and tested. In addition, data from genome wide association studies has also been investigated to explore a potential inherent genetic preponderance to diabetes amongst South Asians. There are also various external factors such as diet and physical exercise, which have also been extensively studied as role players in the pathogenesis of diabetes in South Asians.

1.9.1.1 Insulin Resistance (IR)

It is well known that South Asians have increased insulin resistance in comparison to Caucasians (60). IR is recognised as the predominant factor driving dysglycaemia in South Asians. Insulin resistance is detected amongst South Asians as early as at birth as noted in various studies including Born in Bradford study (10% higher insulin levels despite lower birth weight, in umbilical cord blood amongst South Asian babies compared to European counterparts)(61). This has previously been reported in a study from India as well.

Insulin resistance is also prevalent amongst young adolescents of South Asians descent, as reflected in studies using various parameters.

Eight to eleven-year olds: noted to have higher insulin and higher triglyceride levels (62).
Thirteen to sixteen year olds: higher glucose, insulin and HOMA calculated IR as per Ten Towns Heart Health study (63).
Nine to ten year olds: higher fasting glucose, HbA1c, higher triglycerides and lower HDL levels despite lower weight and lower waist circumference in the CHASE study (64).

1.9.1.2 Beta cell function

It is well known that beta cell dysfunction occurs early in the pathogenesis of diabetes in South Asians (65). However, the mechanisms underlining this early beta cell dysfunction are not fully understood.

1.9.1.2.1 - *Beta cell exhaustion (66)*:

Firstly, one proposed theory is that beta cells in South Asians undergo early exhaustion as a result of prolonged period of compensatory increased beta cell function secondary to early onset insulin resistance.

1.9.1.2.2 - Earlier decline in beta cell function:

Secondly, there is the possibility of earlier decline in beta cell function in South Asians in comparison to White Europeans. The MASALA and MESA studies conducted in the US show lower HOMA-B values in middle aged South Asians in comparison to those of white European, Chinese America, Hispanics and African American descent (67). Beta cell dysfunction has been found to be strongly associated with diabetes and prediabetes amongst South Asians (68, 69) . Another cross sectional study of South Asian Indians, showed evidence of beta cell dysfunction even in the prediabetes stage, irrespective of age, adiposity, family history and insulin sensitivity (65).

1.9.1.2.3 Reduced beta cell function:

The other possibility would be that South Asians inherently have reduced beta cell function. Evidence from the Southall (70) and the Whitehall study (53) demonstrating an increased beta cell capacity amongst South Asians at aged 50, would be inconsistent with this theory. In addition the intrauterine malnutrition theory has been linked to increased insulin resistance rather than beta cell dysfunction amongst South Asians (71) . However, there is a study from Denmark associating low birth weight with reduced beta cell function in later life (72).

1.9.1.3 Ectopic fat deposits

Increased liver fat or non-fatty alcoholic liver disease has been associated with pathogenesis of type 2 diabetes. South Asians have higher levels of liver fat compared to White Europeans (73, 74).

1.9.1.3.1 Pancreatic fat

Some groups have also suggested that ectopic fat deposition around the pancreas may contribute to early beta cell dysfunction (75). However, it has yet to be elucidated if this plays a role in the beta cell dysfunction in South Asians.

1.9.1.3.2 Adipose tissue distribution

South Asians are more prone to adiposity as well as storing more metabolically active fat at comparatively lower BMI. In addition, they also deposit ectopic fat such as in the liver, which is believed to further contribute to the pathogenesis of diabetes.

1.9.1.4 Adipose tissue

There are three depots of adipose tissue namely superficial subcutaneous, deep subcutaneous and visceral fat. While the superficial subcutaneous fat is the primary component, it is metabolically less active than deep subcutaneous and visceral fat. Deep subcutaneous and visceral fat components are metabolically active and related to high transmembrane fluxes and hence associated with dysglycaemia and dyslipidaemia. South Asians saturate their superficial fat stores at a much lower BMI compared to Caucasians (76). As a result they have a propensity to store fat in more metabolic active forms such a deep subcutaneous and visceral fat (76). This is one of the proposed hypotheses (adipose tissue overflow hypothesis) underlining increased risk of type 2 diabetes in South Asians. The hypothesis is discussed in detail in a later section.

1.9.1.5 Adiposity in South Asians

South Asians are known to have more adipose tissue mass at a given BMI in comparison to White European counterparts (77). In addition, there is higher visceral adiposity despite similar BMI, amongst South Asians, in comparison to Caucasians (77). Overall body fat distribution in South Asians is also different with propensity for fat deposition in lower rather than the upper body(42, 76). There is increased ectopic fat deposits in the dorso-cervical and under the chin area, has been reported to be a novel phenotype marker suggestive of increased risk of metabolic syndrome in South Asians (76).

For a given BMI, when compared to Caucasians, peripheral subcutaneous fat is also less in South Asians. This has been noted even amongst young children, when comparing skin fold thickness (78). The morphology of subcutaneous adipose tissue cells in South Asians is also believed to be different compared to Caucasians, resulting in limited storage capacity (73, 79, 80). Varied body fat distribution has been proposed as one of the plausible factors underpinning increased risk of cardiovascular disease and diabetes amongst Indians.
This includes truncal and abdominal adipose tissue deposits.
In comparison, South Asians have reduced superficial subcutaneous fat as noted by comparing skinfold thickness.

There are various mechanistic hypotheses proposed to underpin the varied distribution in fat deposits.

Given the risk of diabetes at a comparatively lower BMI than Europeans, recent international guidelines for screening of 'at risk' population based on BMI threshold, has changed. The American Diabetes Association recommends screening Asian Americans with BMI greater than 23 kg/m^2 (48). The National Institute of Clinical Excellence (NICE) recommends screening 'at risk' South Asians with BMI between 23 to 27.5 kg/m^2 (49).

1.9.1.5.1 Proposed reasons for altered fat distribution with increased obesity in South Asians

1.9.1.5.1.1 Thrifty genotype

This was first proposed in 1962 (81). Neel postulated that certain individuals are programmed with a low threshold to store excess energy to survive potential episodes of food scarcity such as famines. However, this is counterproductive when the same group is exposed to continuous periods of excess nutrition as it results in early onset obesity with other downstream risks including diabetes. This theory has been extensively debated in the recent times. The current view is now in favour of the fact that the thrifty gene theory confers an advantage for procreation rather than survival per se.

1.9.1.5.1.2 Low birth weight/thrifty phenotype

Hales & Barker proposed that individuals with low birth weight were at higher risk of diabetes, dyslipidaemia and vascular disease (82). Lower birth weight, a marker of fetal malnutrition, is more prevalent amongst babies born to South Asian women. More recent understanding suggests that the overall risk of adult onset diabetes is related to the 'catch-up' weight gain that occurs following birth, rather than the actual birth weight per se (83). In addition low birth weight is also prevalent amongst children born to South Asian mothers in the United Kingdom making malnutrition as unlikely causal factor (84, 85).

1.9.1.6 Adipose tissue overflow hypothesis

This was proposed as one of the mechanisms driving increased insulin resistance at a lower BMI in South Asians (86). This is a more recent hypothesis that proposes a relatively smaller capacity amongst South Asians, to deposit adipose tissue in superficial compartments. As a result, these sites when saturated due to increasing adiposity, leads to further adipose tissue deposits in secondary compartments such as subcutaneous and visceral compartments. The deep adipose tissue compartments are more metabolically active with increased transmembrane free fatty acid fluxes resulting in dysglycaemia and dyslipidaemia.

1.9.1.7 Drifty genotype

The theory suggests that, in the absence of predation selection pressure, genes that promote energy storage and obesity were not removed by natural selection and simply were allowed to drift in the genetic journey of human evolution, such that they explain the obesity pandemic in modern western societies. The drifty genotype theory was proposed by Speakman, in contradiction to the previously known theory (87).

1.9.1.8 Genetics

Several GWAS studies have implicated genetic and epigenetic variations as one of the possible reasons for increased susceptibility to type 2 diabetes in certain ethnic groups (88-90).

In addition, given the association of obesity with type 2 diabetes through the recognition of the FTO gene, the question arises if South Asians have a genetic basis for an increased predisposition to type 2 diabetes, in comparison to Europeans.

A meta-analysis by a Canadian group (91) looked at studies of genetic variants associated with type 2 diabetes in South Asians. The group found twenty-four single nucleotide polymorphisms (SNPs) from twenty-one loci associated with type 2 in South Asians. The group found no significant difference in risk estimates between South Asians and Caucasians as a result of SNPs common to both groups. From the eight out of twenty-four novel SNPs found from South Asian genome wide association studies, some also showed nominal associations with type 2 diabetes in Caucasians. As a result, the group concluded that there was lack of strong evidence to date indicating that South Asians had a greater genetic preponderance to type 2 diabetes.

A recent review by Wells et al (92), on the other hand report more up-to-date evidence which suggests a genetic component albeit small, contributing to the increased risk of type 2 diabetes in South Asians. Wells et all report the same Canadian group as above (80), regarding some diabetes risk alleles amongst South Asians but not resulting in an increased diabetes risk amongst Caucasians.

Chambers et al (93) have reported the largest genome sequencing to date, in South Asians. Genes related to energy/lipid metabolism represent one of three key groups of genes that show unique South Asian alleles or allelic stratification compared with

Europeans, along with those associated with immune function and skin/hair pigmentation. Since such genotypes relating to both immune function and skin/hair phenotype can be linked to specific selective pressures affecting South Asian populations, i.e., diseases endemic to the Indian subcontinent and ultraviolet radiation, respectively, it is hypothesized that the metabolism-related variants would be similarly a response to long-term selective pressures.

The same group (94) investigated the potential role of genetic variation driving preponderance to central obesity amongst South Asians. Following genome-wide and exome -wide association analyses as well as risk allele frequency, there was no evidence of any significant ethnic genetic variation associated with the increased risk of obesity in South Asians.

More recently, some genes have been found to have an association with the risk of type 2 diabetes in East Asians including the KCNJ11 gene (95, 96). However, a similar genetic association risk is not found with South Asian subgroups (95).

Therefore, given the evidence to date, one can conclude so far that there is no substantial proof of a strong genetic causal link driving type 2 diabetes in South Asians.

1.9.1.9 Fetal Programming

Lower birth weight has been shown to be associated with increased risk of type 2 diabetes. South Asians are known to have lower weight at birth in comparison to Europeans. However, South Asian babies have a higher percentage of body fat at birth along with higher cord insulin concentrations and greater insulin resistance (78, 97). Lawlor et al however demonstrated *that if the cord leptin concentration was adjusted for the higher maternal fasting glucose levels, then the difference became insignificant in comparison to Caucasian babies* (97). Lawlor et al made this observation amongst children of Pakistani descent (97). The same group also supported the hypothesis that the association of Pakistani ethnicity with greater fat mass at birth, may be mediated by higher maternal fasting blood glucose levels (97).

The recently published Born in Bradford study has shown a positive association between mother's BMI and offspring adiposity amongst Pakistani children but an unclear association with gestational glycaemia (61). This was a prospective study of children born between 2007 to 2010 in Bradford, United Kingdom. A total of 6060 mother offspring pairs (2,717 White British & 3,343 Pakistani) were studied with

offspring data collected at age four to five. The authors noted that gestational glycaemia did not appear to have a clear association with offspring adiposity in either of the ethnic groups.
In addition the Child Heart and Health Study in England (CHASE) has shown that lower birth weight is not accountable for the increased risk of type 2 diabetes in South Asians (98) . Evidence from the CHASE and Born in Bradford Study (61), do not support the hypothesis of a role for fetal programming in the pathogenesis of diabetes in South Asians.

Interestingly the EACH study from Germany (99) looking at pregnancy outcomes in overweight and obese women as well as those with gestational diabetes, failed to show an association between maternal weight or pregnancy weight gain with neonatal birth weight, insulin or leptin levels. Instead the group noted that maternal glycaemia in third trimester was more influential in terms of neonatal outcomes. This is a novel observation. However further studies need to be undertaken amongst South Asians to explore if similar associations can be replicated.
Study by Yajnik et al (100) strongly supports the theory of intrauterine programming and epigenetics as contributory factor in the increasing incidence of diabetes amongst South Asians.

1.9.1.10 Dietary

Traditional South Asian diet is comparatively carbohydrate dense with rice and wheat flour being staple constituents (101). Also, the source of protein in traditional South Asian diet is predominantly plant rather animal based. The role of white rice in the incidence of diabetes has been studied extensively. The glycaemic excursions are noted to be higher following rice intake in South Asians. Recently there has been dietary changes amongst indigenous Indians including consumption of more refined carbohydrates. In addition certain cooking practices common amongst South Asians, such as frying at high temperature with oil reheating resulting in increased trans fatty acids in oils, are also deleterious (102).

Bakker et al have demonstrated unfavourable effects of even short-term overfeeding in South Asians (103). A study from Netherlands comparing responses between healthy and lean South Asian and White European men following just 5 days of high

calorie high fat diet. In contrast to Caucasians, South Asians had increased fasting glucose and insulin levels with reduced insulin sensitivity following this short period of excess calorie consumption.

Amongst migrant South Asians, some further dietary differences have been noted.

Firstly, with time, the food preferences made by migrant South Asians have been observed to be similar to the background population, resulting in higher energy and fat intake in contrast to the traditional South Asian diet (104). The CHASE study has also shown that South Asian children aged 9-10, particularly of Bangladeshi descent have a higher energy, fat and protein intake than white Europeans (105).

However recently there has also been a sea change in dietary habits of endogenous Indians as well, resulting in consumption of high energy and high fat foods in general (106). Therefore, certain dietary effects as well as food choices including cooking practices, appear to contribute to existing increased risk of type 2 diabetes amongst South Asians.

1.9.1.11 Physical activity

Reduced physical activity (107, 108) even independent of BMI (108), is known to be associated with increased insulin resistance. A five-year prospective study of 72,608 Danish adults showed a positive association of increased sitting time with incident diabetes. However, this risk was attenuated by physical activity and was significant only in those falling in the obese category. There is evidence of a dose response association between sitting time and all-cause mortality and cardiovascular disease, independent of leisure time physical activity (109). Increased sitting time is also associated with increased cardiovascular disease.

A recent randomised trial showed improvement in fasting glucose, dawn phenomenon and night time glycaemic variability by interrupting prolonged sitting time by three minutes light intensity walk every 15 minutes, in a group of twelve subjects with type 2 diabetes (110).Increasing physical activity has been shown to reduce the incidence of diabetes even in the absence of weight loss as noted from the diabetes prevention trials (38, 52).

There are many studies suggesting that South Asians particularly living in high-income countries are less physically active than their European counterparts (111). Also, South Asians remain insulin resistant despite adjustment for increased levels of physical activity (111, 112). Cardiorespiratory fitness has been linked to risk of type 2 diabetes as well as an important causality factor (66). South Asians have been found to have lower cardiorespiratory fitness compared to Europeans, unaccounted for by differences in physical activity (111, 112). Relatively reduced physical activity has also been observed amongst young children of South Asian descent as reported by the CHASE study (64). The investigators of the CHASE study compared physical activity amongst 2071 children (aged 9-10) of South Asian, Caucasian and African-Caribbean descent whereby South Asian children had lower objectively measured physical activity (64). Similar observations have been reported from another longitudinal study looking at activity levels amongst children of multi-ethnic background (a total of 281 children of Caucasian, South Asian and Black ethnic group), over a period of 12 months (113). Reduced physical activity in South Asian children could partly be influenced by lack of awareness amongst parents (114, 115) and other cultural influences (114).

The CHASE group also reported reduced levels of physical fitness amongst South Asian children (a total 1625 children of South Asian, Caucasian and black African-Caribbean descent, aged 9-10 years) likely to some extent as a result of reduced physical activity (116). In addition lower levels of physical fitness was associated with higher levels of insulin and insulin resistance (HOMA-IR) amongst South Asian children (117). To achieve comparable levels of cardiorespiratory fitness as White Europeans, South Asians need to engage in greater levels of physical activity and/or have a lower body weight. Based on the evidence, the current physical activity guidelines from India, recommend a much higher level of physical activity for South Asians for diabetes and cardiovascular disease prevention.

Impaired glucose tolerance/Prediabetes

The annualized conversion rate from prediabetes to diabetes has been reported around 13% amongst South Asians.

Investigators from the Chennai Urban Rural Epidemiology Study (CURES), a population-based study in India have reported a very high incidence of diabetes and prediabetes amongst South Asians. South Asians progress from IGT to overt diabetes more rapidly than While Europeans. Data from CURES study estimate an annual conversion rate of 12-18% for South Asians with impaired glucose tolerance (51, 52, 118). In contrast the annual conversion rate from prediabetes to diabetes, amongst Caucasians is 5-11% (119).

1.10 Incretins

Background

Incretins are intestinal hormones secreted from the enteroendocrine cells, released within minutes of food intake secondary to direct and neural stimulation (120). However, this hypothesis is based on findings in rodents and not reproducible in humans. The incretin effect is defined as the amplification of insulin secretion observed on oral glucose ingestion as opposed to when infused intravenously to provide identical plasma glucose concentrations (121). The incretin effect was first described in the 1960s (122, 123).

Le Barre coined the term 'incretin' in the early 1930s (Le Barre 1932) however proof of concept studies with the discovery of the two incretin hormones gained momentum only after availability of radioimmunoassay for insulin (124). The incretin effect is quantified by comparing insulin or C-peptide responses to oral or intravenous glucose loads, adjusted to cause identical increases of plasma glucose concentrations(125, 126). By using C peptide as the surrogate marker of insulin secretion, changes to insulin responses can be quantified independent of the effects of hepatic extraction (127).

Incretin hormones are now known to influence a considerable part of the postprandial insulin response (128). The two gastrointestinal hormones currently known to convey the incretin effect are GLP-1 and GIP (120) constituting the endocrine part of the entero-insular axis as described by Unger et al (129).

Glucose intolerance in mice following deletion of either incretin receptors and studies using GIP and GLP-1 receptor antagonists and mimicry experiments have confirmed the role of incretin axis in normal glucose homeostasis (130).

The insulinotropic effects of GIP and GLP-1 are additive and together fully explain the incretin effect (131). In healthy subjects, the incretin hormones may account for almost 70% of the meal induced insulin response and thereby indirectly facilitates the uptake of glucose in muscle, liver and adipose tissue (126). Nauck et al have also shown the up-regulation of the incretin effect in response to increasing oral glucose loads in healthy subjects (126).

In addition, differences in meal size as well as composition result in varying incretin responses. Whereas fat preferentially stimulates GIP secretion, carbohydrates primarily stimulate GLP-1 secretion in humans (132). The same group also showed that larger carbohydrate load results in a larger GLP-1 release regardless of the glycaemic status.

Both GIP and GLP-1 exert their effects by binding to their specific G- protein coupled receptors, stimulating glucose dependent insulin secretion (133). While extra pancreatic GLP-1 receptors (GLP-1R) are found in the brain, kidneys, lungs, pituitary gland, heart, stomach, small intestine, and major blood vessels, effects of GIP has also been described on various tissues including the central nervous system, adipose tissue, and bone (120, 134).

Both GIP and GLP-1 undergo proteolytic degradation catalyzed by dipeptidyl peptidase-4 (DPP-4) resulting in rapid inactivation (135) *and* hence both intact and total (intact plus DPP-4 metabolised) forms of GIP and GLP-1 must be measured to study incretin secretion and processing in vivo(133). Also possibility of changes in DPP-4 activity according to glycaemic status (136, 137) further stresses the importance of measuring both total and intact forms of incretin hormones.

While actions of GIP are assumed to be fully mediated by GIPR, not all of the documented GLP-1 actions, specifically those of the GLP-1 (9-36) amide metabolite, can be accounted for by activation of the pancreatic GLP-1R, raising the possibility of a putative 'second GLP-1 receptor' (138).

GIP

GIP is a 42 amino acid peptide produced predominantly in duodenal K cells in the proximal small intestine secreted primarily following nutrient intake (139).
Full length GIP (1–42) is rapidly converted to bio inactive GIP (3–42) within minutes of secretion from the enteroendocrine K cells after DPP-4 degradation (135). Hence, circulating immunoreactive GIP represents a mixture of active GIP (1–42) and inactive GIP (3–42) (139).

GLP-1

GLP-1 is a 31 amino acid hormone produced from proglucagon and secreted from L cells present in distal small bowel and colon (140) with enhanced secretion in response to meals or glucose as in case of GIP (141). More recently, GLP-1 has been found to be produced from the pancreas in mice (Chambers 2017) (142).

GLP-1 being susceptible to amidation, circulates in non-amidated GLP-1 (7-37) and amidated GLP-1 (7-36) forms both of which show similar insulinotropic effects and metabolism in humans (143). Although most of the GLP-1 secreted from the gut is amidated in humans (144) , careful considerations are required when measuring GLP-1 levels as some antibodies only recognise amidated GLP-1 (133).

GLP-1 is also rapidly degraded by DPP-4 to GLP-1 (9– 36) amide or GLP-1 (9–37) following release from gut L cells. These metabolites (GLP-1 9-36 and 9-37) account for almost 80% of the immunoreactive GLP-1 in the plasma (145). Although a separate receptor for GLP-1 (9–36) amide has not yet been identified, evidence supports a role for this peptide in regulation of cardiovascular function (146).

Biological actions of GIP and GLP-1

Both GLP-1 and GIP similarly potentiate nutrient-induced insulin release but have differing extra-pancreatic effects(120).

GLP-1

GLP-1's insulinotropic activity, which is strictly glucose dependent (127), is at least partly exerted via interaction with the GLP-1 receptor located on the cell membrane of the beta cells (121) along with a speculated indirect action by activation of vagovagal reflexes (147). GLP-1 promotes insulin biosynthesis, β-cell proliferation and survival (148) and differentiation of exocrine cells or islet precursors toward a more differentiated β-cell state (149). GLP-1 strongly inhibits glucagon secretion through a likely indirect mechanism (150).

Besides the insulinotropic effect, GLP-1 also inhibits gastric emptying via both central and neural effects (120). GLP-1 inhibits appetite and food intake in normal subjects as well as in obese subjects with T2DM and it is thought that GLP-1 is one of the gastrointestinal hormones that normally regulate food intake (127).

GLP-1 also has cardiovascular effects mediated directly through receptor activation. Overall effects include those on left ventricular function, coronary blood flow (146) and protective effects following ischemia (151) primarily in murine experiments. Some of the cardiac effects may be mediated by the GLP-1 9-36 amide, a degradation product formed rapidly in circulation (127). Corroborating evidence from human studies especially in terms of effects on left ventricular function (152), myocardial oxygen uptake (153) and endothelial function (154) is now emerging.

GLP-1 also influences energy expenditure (120, 155). GLP-1 receptor knockout mice have cortical osteopenia, bone fragility, increased osteoclast numbers and markers of bone resorption (156) suggesting a role of GLP-1 in modulating bone physiology.
There is also some evidence that GLP-1 may possess neurotropic effects leading to a possible role in treatment of neurodegenerative diseases such as Alzheimer's disease (157) as well possible role in neurogenesis in Parkinson's disease (158).

GIP

Unlike GLP-1, which exerts multiple non-incretin activities in the regulation of blood glucose, the primary action of GIP is the stimulation of glucose-dependent insulin secretion.

In contrast with GLP-1, GIP does not delay gastric emptying in humans, and nor does it inhibit glucagon secretion. On the other hand, glucagon may be stimulated by GIP under certain conditions (120). GIP has been shown to promote β-cell proliferation and cell survival in islet cell line studies (159).

GIP also plays a role in adipocyte biology with experimental evidence indicating that GIP regulates fat metabolism in adipocytes, including enhanced insulin-stimulated incorporation of fatty acids into triglycerides, stimulation of lipoprotein lipase activity and stimulation of fatty acids synthesis (120). GIP also promotes energy storage via direct actions on adipose tissues(120).

Endogenous GIP also regulates bone metabolism as evident from studies in GIP receptor knockout mice resulting in high turnover osteoporosis (160). In addition GIP has direct anti-apoptotic action on osteoblasts in vitro, suggesting a role of GIP in stimulating bone formation (160).

Table 0.1 Summary of the main characteristics of GIP and GLP-1(161)

	GIP	**GLP-1**
Characteristics		
Peptide	42 amino acids	30 amino acids
Released from	K cells: duodenum	L cells: ileum and colon
Active form	Single bioactive form	Two bioactive forms: (7-37) and (7-36) amide
Inactivated by	DPP-4	DPP-4
Physiological actions		
Insulin secretion	Stimulated	Stimulated
Insulin biosynthesis	-	Stimulated
Beta cell proliferation	Promoted	Promoted
Glucagon secretion	-	Inhibited
Food intake	-	Reduced
Gastrointestinal motility	-	Participates in the ileal brake
Cardiac function	-	Improvement
Bone resorption	Inhibition	Inhibition
In type 2 diabetes		
Secretion	Normal	Reduced
Response	Impaired	Preserved

Measuring incretin hormones

For the purpose of this thesis the term incretin hormones are used collectively in reference to GLP-1 and GIP only (unless otherwise stated). Incretin hormones are labile with a very short half-life due to enzymatic degradation in vivo. At any one time, depending on species and the feeding status, more than one form of both GIP and GLP-1 can be detected in plasma (162). However not all these various forms are biologically active or even insulinotropic. Hence, for accurate interpretation of incretin hormone levels and drawing inferences, it's important have an understanding of the tests used to measure these hormones.

1.10.1.1 'Active' and Total levels

GLP-1 is a 31 amino acid hormone produced from proglucagon and secreted from L cells which are present in distal small bowel and colon (140). Following post-translational modification, GLP-1 gets converted into an amidated peptide (GLP-1 7-36) and a glycine extended peptide (GLP-1 7-37). Both forms show similar insulinotropic effects and metabolism in humans (143) and are referred as total GLP-1. They are also susceptible to enzymatic degradation by DPP-4 which is located in the luminal membranes of the vascular endothelium (163) as well as circulating in plasma (164). GLP-1 is later degraded to GLP-1 (9-36) or GLP-1 (9-37) following enzymatic cleavage. The degraded products are called 'intact' GLP-1 and account for 80% of the immunoreactivity in the plasma (145). The circulating half-life of GLP-1 (7-36) and GLP-1 (7-37) is only minutes, resulting in higher circulating forms of truncated peptides than undegraded forms in humans (145).

Both GLP-1 (7-36) and GLP-1 (7-37) appear to have the same insulinotropic effect (143). The N terminal is crucial for biological action while the C terminal is required for receptor binding (143).

Both GLP-1 (9-36) and GLP-1 (9-37) are N terminally truncated. This renders them biologically inactive but with the ability to bind to the receptors. Hence, they can also have antagonistic action by competitive receptor binding. Although a separate receptor

for GLP-1 (9–36) amide has not yet been identified, evidence supports a role for this peptide in regulation of cardiovascular function (146).

Circulating forms of GLP-1 including GLP- 1(7-37), GLP-1 (7-36), GLP-1 (9-37) and GLP-1 (9-36) vary based on species and the feeding status (162). Therefore, it's important to know what is actually measured through the tests used in studies before we can fully understand the reasons behind variation in GLP-1 responses under different conditions.

1.10.1.2 Immunoassays

The immunoassays for measuring incretin hormones use antisera raised against different peptides. However, as mentioned above, there are various forms of incretin hormones in circulation, at any one time. This leads to potential cross reactivity, resulting in inaccurate measurement of incretin hormone levels. However, development of more specific assays using smaller peptide fragments, has led to more accurate and robust hormone measurements. When measuring 'active' GLP-1 or GIP, use of DPP-4 inhibitor while collecting blood samples, is recommended. Levels of 'active' GIP and GLP-1 levels without use of DPP-4 inhibitor, would be clearly affected by degradation.

1.10.1.3 Radioimmunoassay (RIA)

This is a heterogenous assay that employs a radiolabeled antigen. A radiolabeled target antigen is then bound to a known amount of a specific antibody. It's then mixed with an unknown amount of the antigen present within a sample (serum/plasma). The unlabeled antigen(cold) from the sample competes with the radiolabeled antigen(hot) for antibody binding sites. As the concentration of the 'cold' antigen is increased, more of it binds to the antibody and displaces the radiolabeled antigen. The bound antigens are then separated from the unbound ones and the radioactivity of the free remaining unbound antigen in the supernatant is measured (165).
Direct plasma measurements by RAI without extraction, had led to unreliable reading due to other factors in the plasma resulting in assay interference.

1.10.1.4 Extraction (ethanol or solid phase)

This helps to reduce non-specific interference during incretin hormone levels estimation. Introducing an extraction step during estimation of incretin hormone levels, helps to reduce variability and increase reproducibility.

1.11 Incretins and Pathophysiology

Incretins and obesity

Obesity has consistently been shown to be associated with an impaired incretin effect independent of the effect of glucose tolerance (166), likely due to associated insulin resistance. Also, insulin resistance on its own has been found to be associated with impaired postprandial incretin responses (167).

Incretins and Diabetes

Abnormalities in the incretin axis have been shown to play an important role in the progressive beta-cell failure in T2DM and hence defining the contribution of the gastrointestinal tract in the pathogenesis of the disease (39). Various aspects of incretin biology have been researched, to elucidate their role in the pathogenesis of type 2 diabetes. The potential role of incretin dysfunction in the aetiology of type 2 diabetes also remains widely debated.

1.11.1.1 Incretin secretion (GLP-1 and GIP) in type 2 diabetes

In terms of effects of diabetes on individual hormone secretion, the evidence remains rather inconclusive.

GIP

GIP levels in T2DM were reported as increased, decreased or unchanged in previous studies (168). However a more recent study using newer GIP assays found slightly decreased GIP levels in a large group of T2DM patients in comparison to a matched group of subjects without diabetes (169). Overall a major GIP secretory defect does not appear to be associated with T2DM (128).

GLP-1

In contrast, GLP-1 secretion was found to be significantly impaired in T2DM especially in the late postprandial phase (169). However decreased GLP-1 response does not appear to be a consistent finding in all studies (141, 170).

In conclusion impaired secretion of incretin hormones may not be a constant finding in T2DM but a decreased secretion of GLP-1 after mixed meals is observed in most studies (171).

Hormone Responses (Incretin effect)

A reduced incretin effect in T2DM was first described in 1987 (172). In addition, in subjects with type 2 diabetes there is also a reduced ability to up-regulate the incretin effect in response to the increasing oral glucose loads (173). Loss of incretin effect is now recognized as a specific, important and an early characteristic of T2DM (128). A recent prospective study looked at the relationship between GLP-1 responses and fasting blood glucose levels in relation to dysglycaemia, over a period of 7 years. The study adds to the evidence that incretin dysfunction precedes development of overt diabetes.

However the reasons underlying the loss of incretin activity in T2DM are still incompletely understood (174).

1.11.1.2 Insulinotropic effects of incretin hormones in type 2 diabetes

GIP

In terms of the actual effects of incretin hormones on pancreatic islets, the insulinotropic effects of GIP are found to be lost in T2DM (175). Even supraphysiological levels of GIP have little effect on insulin secretion as demonstrated in a seminal study by Nauck et al (176) though restoration of insulinotropic effect after four weeks of sustained euglycaemia has been shown (177). Interestingly, the loss of GIP activity seems to be more pronounced during continuous infusion than after an intravenous bolus administration of the peptide (174). The loss of insulinotropic action of GIP in T2DM is thought to be due to a general impairment in beta cell function along with possible down regulation of the GIPR on account of hyperglycaemia (174, 178). The lack of glucose lowering activity of GIP in T2DM may partly be also related to its stimulation of glucagon release (179), which counteracts its residual glucose lowering actions.

GLP-1

In the same seminal study by Nauck et al (176), GLP-1 infused to attain slightly supraphysiological concentrations, had profound effects on insulin secretion and actually normalised the insulin response to a mildly hyperglycaemic clamp in a group of patients with type 2 diabetes. The preserved insulinotropic effect of GLP-1 with loss of effect of GIP in T2DM has been further proven in a large study by Vilsboll et al (180).

A recent meta-analysis has suggested that there is not an universal progressive decline in the GLP-1 response in patients with type 2 diabetes, rather it may be predicted by individual characteristics such as age, weight, fasting non-esterified fatty acid (NEFA) levels and glucagon levels (171). It appears that besides association of poor GLP-1 response with duration and severity of diabetes, BMI also remains a significant determinant (181). The effects of obesity on incretin hormones is discussed in detail below.

1.11.1.3 Cause or Effect

As far as cause and effect are concerned, this remains a focus of on-going debate. Evidence so far is not entirely consistent. Impaired GLP-1 secretion appears to be the consequence rather than the cause of diabetes (127). To improve understanding of the timeline of incidence of incretin dysfunction, in the pathogenesis of type 2 diabetes, there have been various studies amongst individuals with a known increased risk of type 2 diabetes.

First degree relatives of those with type 2 diabetes:

Normal GLP-1 and GIP secretion post oral glucose load has been reported in first degree relatives of people with type 2 diabetes (182).

Gestational diabetes:

Pregnancy results in a degree of insulin resistance. Gestational diabetes is a strong predictor of type 2 diabetes in the future. Women with family history of diabetes, pre gestational obesity and advanced age at conception, are at increased risk of gestational diabetes.

There have been some studies on individuals with former gestational diabetes to understand if an incretin defect may be an epiphenomenon of diabetes.

- **Meier et al 2005(183)**

  Meier et al studied subjects with previous gestational diabetes (6 months) in comparison to control subjects (age and BMI matched individuals with history of recent pregnancy). This group found no differences in insulin secretion in response to GIP administered by continuous infusion during a hyperglycaemic clamp or as an intravenous bolus in the fasting state between women with previous gestational diabetes and control subjects. Likewise, GLP-1 and GIP levels after oral glucose ingestion were normal in women with previous gestational diabetes.

- **Pacini et al 2012(184)**

  This group measured the incretin effect in one hundred and four women (104) with formal GDM (fGDM). Control subjects included thirty-five (35) women with recent pregnancies. The OGTT included a 75-gram glucose load. The fGDM group was further subdivided into those with normal versus those with impaired glucose tolerance. Interestingly this group did show impaired beta cell function in individuals with fGDM. However, the study was an exception to others reported so far, as the OGTT and intravenous GTT did not show isoglycaemic patterns. Also, they did not measure incretin hormones.

Meier et al (183) findings conforms with the current viewpoint that a diminished incretin effect is likely an epiphenomenon of chronic hyperglycaemia (174).However it may be too early to conclude that advancing type 2 diabetes is characterised by a progressive loss of the potential to secrete GLP-1 as part of the process of disease progression (171). Also reduced GLP-1 levels in type 2 diabetes do not appear to be a universal characteristic representative of all patients and seem to be influenced by various factors including age, body weight (obesity), fasting NEFA concentrations and fasting glucagon concentrations (171).

1.12 Glucagon

Relative or absolute hyperglucagonaemia in fasting and post-prandial states remains a key contributor to the pathogenesis of type 2 diabetes. In the post prandial state, both incretin hormones (GLP-1 and GIP) have opposing actions on glucagon levels. The close interplay between glucagon, incretin hormones and tightly regulated glucose levels has been reviewed recently with significant interest.

Measurement of glucagon levels

Similar to other incretin hormones, measurement of peripheral plasma concentrations would not be truly indicative of glucagon secretion. The reasons for this are multifold. Firstly a proportion of the glucagon secreted undergoes hepatic clearance (185). Secondly its precursor, proglucagon, undergoes pancreatic and intestinal processing. However the resulting sequence of glucagon varies in both cases (186). Therefore, to estimate alpha cell derived glucagon secretion rate per se, its measurement should be based on assays that are sensitive for detecting post pancreatic processing sequences. In addition, as glucagon levels can be suppressed to very low levels such as 2-3 pmol/l, detection limits of assays would need due consideration.

1.12.1.1 Glucagon physiology

Circulating glucagon levels in plasma are low with reported average level being 10 pmol/L (169). In the fed state, glucagon levels are known to achieve a nadir value of 2-3 pmol/l and rise to almost 40 pmol/l in the setting of hypoglycaemia.

1.12.1.2 Glucagon and incretins

GLP-1 has been identified as a powerful suppressor of glucagon secretion while GIP has a stimulatory effect. On the other hand, GLP-2 has also been found to stimulate GLP-1 secretion. Due to these opposing actions, the net effect of incretin hormones on glucagon secretion is difficult to predict.

1.13 Incretin hormones in non-Caucasian population

Almost all of the landmark incretin studies have been performed in Caucasians (European descent). Recent data does suggest variation in the incretin system based on ethnicity. However, the data is conflicting in the few studies published so far. In addition, the studies so far include different non-Caucasian subjects including Far East Asians, Africa-Americans and South Asians. While all these three ethnic groups are known to be at a higher risk of type 2 diabetes, the underlying pathogenesis of diabetes does vary in each of the groups. While South Asians and Africans Americans are known to be primarily insulin resistant, Far East Asians demonstrate a primary insulin secretory defect.

Hence for these reasons, it would be inaccurate to extrapolate findings from incretin-based studies from Far East Asians to South Asians.

Summary of incretin hormone studies in non- South Asian populations

Emerging evidence amongst individuals from Far East Asia are alluding to a lack of incretin dysfunction associated with dysglycaemia.

1.13.1.1 Mechanistic studies

Moller et al 2014 (187)

This was one of the biggest incretins based mechanistic study amongst non-Caucasians. This was a cross-sectional study comparing Caucasians and Japanese individuals with varying degree of glucose tolerance ranging from NGT, IGT to overt type 2 diabetes.

A total of 150 individuals from North European background were compared with 120 of those of Japanese descent. The study showed similar disposition indices in Japanese and Caucasians , concluding that differences in body composition would likely to explain the differences in insulin sensitivity and beta-cell responses between both groups(187).

Yeow et al (188)

A study amongst young Malaysians (multi-ethnic group), where twenty-five individuals with type 2 diabetes were compared with fifteen age, ethnicity and gender matched controls. The study was amongst very young participants (less than 25 years of age) with recently diagnosed type 2 diabetes (23.1 +/- 6.42 months). All subjects underwent OGTT (75 gm.) and IVGTT (300 mg/kg) on two separate occasions. The investigators demonstrated that total GLP-1 responses were similar between young individuals with type 2 diabetes and controls ($tAUC_{0\text{-}90;}$ 1223.4+/- 79.4 vs. 1347.5+/- 125.84 pmol/L; p=0.385). However, subjects with type 2 diabetes included in this study had been on metformin (omitted at least 5 days prior) and hence a residual effect of Metformin on GLP-1 levels can't be excluded. The authors estimated the incretin effect based on differences in beta-cell responses derived from C peptide measurements between stimulation following oral and intravenous glucose. The study did demonstrate a reduced incretin effect amongst those with type 2 diabetes compared to controls (12.1+/-8.93% vs. 70+/-4.03%, p<0.001). However, the method used for estimating the incretin effect in the study was different from the

'standard' measures including an isoglycaemic clamp study. There was also a lack of compensatory beta cell response to insulin resistance, as demonstrated by a reduced disposition index. The authors conclude that this may partly be due to an impaired incretin effect without a reduction in the total GLP-1 levels.

Chang 2014 (189)

This is a study from Taiwan looking at 202 women with PCOS and 47 healthy controls. The investigators aimed at interrogating potential role of aberrant irisin and GIP metabolism in the development of PCOS. While fasting GIP levels were comparable in both groups, levels at 60 minutes during a 75-gm. OGTT were significantly higher amongst women with PCOS (PCOS 82.57 ± 6.49 pg/mL vs. control, 49.68 ± 5.98 pg/mL, P = .013). The difference in GIP levels persisted even in the subgroup analysis after matching for BMI and metabolic profile.

In contrast, GLP-1 levels remained comparable between both groups (PCOS: 166.85+/-8.47 pM vs. controls: 173.57+/-21.51pM; p=0.74). In addition, the study reports a negative association between stimulated GIP levels at 60 minutes with adiponectin and $ISI_{Matsuda}$.

Fasting irisin levels (P < .001) and glucose-induced GIP response (P = .013) in PCOS subjects were significantly elevated as compared to those of control women(189).

Based on these findings, authors suggest a potential role of GIP in the development of PCOS and further downstream complications.

Park et al 2016 (190)

Investigators studied the association between incretin hormones and newly diagnosed type 2 diabetes in adolescents and obese children from Korea. A total of twelve obese children with type 2 diabetes were compared with age (mean 13.8+/-2 years), BMI and gender matched twelve children without diabetes. Responses post 75 g OGTT (1.75 g/kg body weight) were compared between both groups. Total GLP-1 levels were higher in the diabetes group, however intact GLP-1 and GIP levels were compared between both groups. Total GLP-1 levels (incremental AUC) were higher in the diabetes group (552.1+/- 138.3 vs. 54.3 +/- 33.9 pmol/l, p=0.001)), however comparable iGLP-1 and GIP levels were demonstrated. Total GLP-1 levels were highest at baseline in the normal glucose tolerant group, in contrast to findings from other studies. Investigators did show an inverse relationship between BMI and GLP-1 levels.

1.13.1.2 Drug efficacy studies including meta-analysis

Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS)- East Asians had the greatest initial glycaemic reduction of HbA1c; the absolute fall was 0.6 and 3.9 mmol/mol (0.1% and 0.4%) more than in other groups (191).

It has been shown that DPP-4 inhibitors and GLP-1 receptor analogues have greater glucose lowering efficacy in Asians (192) (193) (194). Given the fact that the primary defect in diabetes amongst Far East Asians is beta cell dysfunction, using incretin-based drugs to ameliorate this, potentially results in a greater glycaemic lowering response. Evolving evidence supports sustained glucose lowering effects of DPP-4 inhibitors in Far East Asians as noted from post hoc age-stratified analysis of the subjects who received sitagliptin therapy for 2 years in a study to evaluate its efficacy and safety for elderly T2DM patients, supports (195).

Incretin hormones in South Asians

Incretins are not well studied so far amongst South Asians. Very preliminary evidence does suggest that the incretin system may vary in South Asians.

1.13.1.3 Why may it be different?

Insulin resistance-

South Asians have evidence of insulin resistance from very early years in life. As we know, even cord levels of insulin have been found to be higher in South Asian babies compared to Caucasians.

Incretin responses have been found to be impaired in the presence of insulin resistance and hence raising the possibility that a similar impairment may be evident in South Asians.

Obesity-

Again, South Asians are known to have higher levels of visceral adiposity for the same BMI, in comparison to Caucasians. Obesity is also known to influence the incretin hormone responses. This has been demonstrated in both murine and human models

The factors mentioned above are key players in the pathogenesis of type 2 diabetes in South Asians. There is already evidence to demonstrate their influence on the incretin hormones and consequently on their glucoregulatory actions. Hence based on this, we hypothesise that the incretin system would vary in South Asians. However, if this variation exists, whether it would be a consequence or a cause of all other known metabolic distinctions in South Asians, is yet to be ascertained.

The evidence so far is summarized as below:

1.13.1.4 Mechanistic studies

Sleddering et al 2014 (196)

This recent study demonstrated higher GLP-1 and insulin levels after a glucose load in young healthy South-Asians living in Netherlands, compared to Caucasian counterparts.

GLP-1 levels preceded the peak insulin response and paralleled the insulinogenic index, thereby suggesting a direct relation between the increased GLP-1 response and insulin secretion by the β-cell.

1.13.1.5 Drug efficacy studies

Various recent studies have demonstrated higher glycaemic efficacy and weight loss with Liraglutide amongst type 2 diabetes patients in India, compared to outcomes reported from large randomised controlled studies in the western population (197). In terms of efficacy, compared to evidence from six phase 3 global randomised liraglutide effect and action in diabetes studies (maximum – 1.5% in LEAD-4 study)(198), data from Indian studies report a HbA1c reduction ranging from 0.74% (199) to up to 2.54% (Chaudhuri 2016) with varying doses of Liraglutide.

Yang et al (200)

A 16-week double blind randomized study (*n* = 929) suggested similar glucose lowering with Liraglutide among all Asians (Chinese, Koreans and Indians).

Kozlovski et al (Vildagliptin) 2016 (201)

This was a post-hoc exploratory analysis of data from twenty trials with vildagliptin, undertaken between the period of 2005 to 2013. The pooled study group included 2746 Caucasians and 2232 Asian patients with type 2 diabetes. Data from Asians was subdivided in three further groups i.e. Chinese (645), Japanese (1005) and Indians (436). Overall similar efficacy was seen in Caucasians (0.68% ± 0.03%) and Asian (−0.80% ± 0.03%) patients (P value for interaction = .56). However, there was a small difference showing improved efficacy in Japanese patients in comparison to Caucasians when analysed by ethnicity ($p<0.02$). The authors propose various reasons for this difference amongst Japanese patients including the lack of placebo effect.

1.13.1.6 Meta-analysis

Park et al Pharmacotherap 2012 (202)

A meta-analysis undertaken from 62 randomised controlled trials suggested a significantly better glucose lowering effect of DPP-4 inhibitors in Asians compared to non-Asians.

How do East Asians differ from South Asians?

An extensive review by Seino's group has looked at various pathophysiological factors that may be responsible for increased efficacy of incretin drugs amongst East Asians (203). Based on studies from the same group as well as recent evidence, authors report impaired early phase insulin secretion amongst Japanese in comparison to Caucasians, throughout all phases of glucose tolerance. Similarly, reduced insulin secretory capacity has been noted in other Far East Asians groups such as Koreans and Taiwanese. In addition, there is greater preservation of insulin sensitivity even in the context of type 2 diabetes, in comparison to Caucasians. However just as South Asians, they do have a greater level of visceral fat at a comparatively lower BMI.

Summary of evidence on incretins in East Asians

- No differences in secretion of GLP-1 and GIP between NGT subjects and those with T2DM, when studied amongst Koreans and Japanese
- Negligible meal induced GLP-1 secretion amongst Japanese subjects
- Lower GLP-1 secretion post 75-g OGTT in Japanese in comparison to Caucasian subjects (contemporaneous measurements as using same assay methods)
- Very low levels of active GLP-1 in Japanese subjects
- Unimpaired incretin effect in Japanese and Korean patients with type 2 diabetes
- Genetic variant of GIP receptor gene
- Dietary habits: the HbA1c lowering activity of a DPP-4 inhibitor has been shown to be enhanced by consumption of fish.

-

1.13.1.7 DPP-4 activity/inhibition

In this section, I explore the evidence around the plausibility of ethnic variation in DPP-4 activity or/and consequent inhibition. In the discussion above, the various studies affirming increased comparative efficacy of DPP-4 inhibitors amongst Asians have been highlighted. However, the reasons underpinning this difference compared to Caucasians, is yet to be elucidated.

There remains the question whether DPP-4 inhibitor activity may vary in Asians and hence account for any potential changes in circulating incretin hormone levels or efficacy of incretin-based drugs. It is also known that DPP-4 levels correlate with adipocyte size and metabolic syndrome (204).

While there are no direct studies measuring DPP-4 activity in South Asians, there has been a recent paper from India looking at the pharmacokinetics and pharmacodynamics (PK/PD) of a single dose of Sitagliptin, a DPP-4 inhibitor, amongst healthy Indians. Sangle et al (205) reported the PK/PD parameters of single dose Sitagliptin in healthy individuals of Indian origin. The group reported >80% DPP4 inhibition which is comparable to previous reports from Caucasian studies (206). Interestingly, post meal weighted average of active GLP-1 levels measured 2 hours after Sitagliptin, was 4.4 fold higher amongst Indians, compared to a previous Caucasian study (206). Post meal GLP-1 levels, however were still within physiological range.

DPP-4 inhibitors appear to have similar PK and PD profile in Japanese individuals in comparison to Caucasians (207, 208).

A recent meta-analysis of efficacy of DPP-4 inhibitors amongst non-Caucasians, has focused more on the pharmacokinetics and DPP-4 inhibition rate (209).

The authors have investigated whether there are ethnic differences in the efficacy of DPP-4 inhibitors between Japanese and non-Japanese patients with T2DM, focusing on the relationship between DPP-4 inhibition and the efficacy of the response to the inhibitor. The HbA1c reduction by DPP-4 inhibitors at each DPP-4 inhibition level was larger in Japanese studies than in non-Japanese studies and a statistically significant interaction of study origin (Japanese/non-Japanese) was observed ($P < 0.0001$).

However the meta-analysis was unable to conclude that presence increased endogenous DPP4 activity amongst Japanese individuals, is responsible for increase efficacy of DPP4 inhibitors, as speculated by some researchers(210).

A recent study by Anoop et al, reports high levels of circulating DPP-4 in non-obese Asian Indians with type 2 diabetes (211). The study included 93 subjects with recently diagnosed type 2 diabetes (less than 1 year) with 40 age and BMI matched normoglycaemic controls. Subjects with diabetes were on metformin only.

Relationship of metformin with incretin hormones

1.13.1.8 Are they more efficacious in South Asians?

As metformin is known to influence circulating GLP-1 levels, it is now increasingly being recognised as a sensitiser and enhancer for incretin-based drugs. There is also intense research around cardioprotective role of metformin that may also be GLP-1 mediated. In this section, I have discussed some of the relevant data around metformin and incretin hormones.

The effect of metformin on post prandial GLP-1 levels in patients with type 2 diabetes, was first reported in 1998 (212). Since then there have been various human studies exploring the interaction of metformin with incretin hormones, with or without incretin -based drugs (213-220).

One of the more recent studies have demonstrated that the effects of metformin therapy on circulating GLP-1 levels are sustained over time (even until 18 months) and independent of changes in weight or glycaemia (221). Preiss et al performed an ancillary study based on the CAMERA (Carotid Atherosclerosis: Metformin for Insulin Resistance) trial (222) and a cross sectional study from the Diabetes Research on Patient Stratification (DIRECT) consortium (223).

The CAMERA trial was aimed at investigating the effect of metformin on surrogate markers of cardiovascular disease in patients without diabetes aged 35 to 75, with established coronary heart disease and a large waist circumference (≥ 94cm in men, ≥80 cm in women). Patients were randomised 1:1 to metformin 850 mg or placebo twice daily. Samples were drawn every 6 months until 18 months. Metformin therapy led to a 25% increase in total GLP-1 levels following 18 months of daily metformin therapy in individuals without diabetes but elevated WC (221). The effects were sustained throughout the duration of study and not influenced by level of adiposity or changes in glycaemia.

The DIRECT (DIabetes Research on Patient StraTification) study is part of a European Union Innovative Medicines Initiative project, with the overarching aim to discover and validate biomarkers of rapid diabetes development, progression and drug response (223). For investigating metformin effects on GLP-1, cross-sectional data were analysed from the baseline visit in 775 participants from work package 2 (aimed to identify predictive biomarkers of glycaemic deterioration, deep phenotyping and

biochemical assays in subjects recently diagnosed with T2D who had been on either metformin or life-style therapy alone at baseline). All patients underwent a MMT (250 ml of Fortisip drink; 18.4 g CHO/100 ml.) test after stopping metformin for 24 hours. Samples were drawn at time 0 and then every 30 minutes until 120 minutes. Metformin users had higher basal, fasting, active GLP-1 levels (+25.5% [95%CI 17.0-35.5%], p<0.001) and fasting total GLP-1 levels (+14.5% [95%CI 8.4-21.0%], p=0.0097) than individuals who were on lifestyle therapy, but not higher incremental GLP-1 levels. Investigators also proposed a potential cardio protective role of metformin given its effects on GLP-1 levels, even in the absence of diabetes. This would be particularly relevant given data from two recent cardiovascular outcome trials (224, 225) as well as a Mendelian randomisation study of a GLP-1 receptor variant associated with lower fasting glucose levels which are also in turn associated with lower cardiovascular risk (226).

Metformin and incretin hormones

Existing literature suggests that metformin interacts directly with the incretin axis acting as an enhancer of GLP-1 secretion and perhaps as a sensitiser, beyond its known biological functions (227). The specific mechanism by which metformin exerts its effect on the incretin axis is not fully understood. It was initially suggested that metformin inhibits DPP-4 activity although not substantiated by other groups (220).

1.13.1.9 Mechanism of action

Data from murine experiments allude to the role of metformin in modification of various components of the incretin axis via pathways dependent on peroxisome proliferator –activated receptor alpha (228). Maida et al have shown that metformin acutely and selectively increases levels of GLP-1 and not other incretin hormones, with no effect on plasma DPP-4 activity in mice. It has also been shown to increase the effects of GLP-1 and GIP in insulin secretion from murine beta cells (228). In humans, various mechanisms of actions have been proposed. However, there are two key hypothesis which are well substantiated. Firstly, it has been hypothesised that metformin may act as a potential secretagogue. Secondly metformin may act as a DPP-4 inhibitor resulting in increased circulating time for endogenous GLP-1. Both these hypotheses are discussed at length by Knop's group (229). The authors cite

various studies favouring the hypothesis that metformin's interaction with gut microbia leads to a consequent increase in GLP-1 secretion, independent of DPP-4 inhibition.

Glucagon and incretin hormones

Patients with T2DM have fasting hyperglucagonaemia as well as exaggerated glucagon responses to meal ingestion (169). It has also been demonstrated that lack of suppression of glucagon contributes to post prandial hyperglycaemia (230).

GLP-1 also strongly inhibits glucagon secretion and this effect may be as important as the insulinotropic effects (231) while glucagon maybe stimulated by GIP in certain conditions (120).

1.14 Gaps in current knowledge

The incretin studies in Asians including South Asians, so far are not consistent in their findings. The following reasons may explain the variation in outcomes from studies so far.

1. Comparison of responses between healthy and diabetes group by using 75 gm. glucose load. This is a strong stimulus especially for the group with type 2 diabetes. Previous landmark studies on incretins amongst Caucasians, have used a smaller stimulus of 50 grams.

2. The limited studies done so far vary in terms of measurements of incretin hormones i.e. total or active GLP-1 or GIP. This influences interpretation of measured incretin hormone responses and certainly not appropriate for comparing findings across various studies.

3. In addition, there is variation between studies, in the methods used for measurement of incretin hormones. These again, have huge implications especially when conclusions are drawn based on comparison with historical rather than contemporaneous Caucasian data.

4. Most of the studies did not simultaneously measure gastric emptying, which remains a key factor in influencing endogenous incretin responses. Varying gastric emptying would be a potential confounding factor.

5. Finally, most of the evidence around incretin biology in Asians has come from Far East Asia especially Korea and Japan. However, in contrast to South Asians, people with type 2 diabetes of Korean and Japanese descent, are comparably leaner with primarily an insulin secretory defect than insulin resistance.

Hence the mechanistic pathway of role of incretins and other gut hormones in pathogenesis of type 2 diabetes, in South Asians remains relatively unexplored.

1.15 Potential implications

It's known that the incretin system is an early and key player in the pathogenesis of diabetes (39). Incretin based drugs are now powerful agents in our current armamentarium of drugs used in type 2 diabetes. These agents particularly GLP-1 receptor analogues have the potential to cause significant HbA1c reduction along with additional non-glycaemic benefits.

South Asians are one of the most 'at risk' groups with a much more aggressive form of diabetes presenting at a much younger age at lower levels of obesity.

Asians including South Asians have been found to have a differing response to incretin-based drugs, in comparison to Caucasians. There is also some limited preliminary evidence to suggest an increased endogenous DPP-4 activity amongst Asians. Therefore, overall there is some emerging evidence suggesting that the incretin system may vary based on ethnicity.

If the incretin system were proven to be different in South Asians, and more so if consequently incretin-based drugs are proven to have an 'ethnicity specific' therapeutic effect, then this would have a significant impact in furthering personalised medicine in management of type 2 diabetes.

1.16 Summary

Compared to Caucasians, African- American subjects have been shown to exhibit higher basal and glucose-stimulated concentrations of total GLP-1, persisting 6 months after treatment with a long-acting somatostatin analogue (232). One Korean and two Japanese studies were published showing that there were no differences in incretin secretion between subjects with normal glucose tolerance, impaired glucose tolerance and newly diagnosed type 2 diabetes, and between obese and non-obese patients with diabetes (233-235). In addition, one Japanese study showed little enhancement in meal induced GLP-1 secretion despite a robust GIP response, when comparing subjects with T2DM and healthy controls (236). The same group showed low intact GLP-1 levels in T2DM group and healthy volunteers alluding to a possibility of selective accelerated DPP-4 processing of GLP-1 amongst Japanese subjects. However in contrast to South

Asians, people with T2DM of Korean and Japanese descent, are comparably leaner with primarily an insulin secretory defect rather than insulin resistance (237).

Currently studies looking at the incretin effect in South Asians in comparison to Caucasians are sparse. There is some suggestion of disparity in response to incretin therapy between Asians and Caucasians. DPP-4 inhibitor Sitagliptin was found to have greater efficacy (glycaemic) in Asian Indians and Koreans (238) compared to Caucasians (239-242).

Chapter 2

Indications, Aims and Hypothesis

Indications, Aims and Hypothesis

1.17 Indication for current research

Evidence so far alludes to a potential variation in the endogenous incretin system amongst South Asians in comparison to Caucasians.
These differences may involve some or all-key aspects of the incretin system, as below:

- The incretin effect in South Asians may be reduced compared to Caucasians

- Meal induced incretin responses may vary amongst South Asians compared to Caucasians

- The endogenous incretins in South Asians may be susceptible to increased DPP-4 inactivation resulting in reduced circulating 'active' incretin hormones

- The insulinotropic response to infused incretins (GLP-1 and GIP) may vary amongst South Asians compared to Caucasians.

1.18 Aims and Objectives

Objectives

We aimed to study some of the above aspects of the incretin system amongst glucose tolerant migrant South Asians in comparison to Caucasians. The purpose for including glucose tolerant South Asians in contrast to those with diabetes were as follows:

- There is evidence to suggest that disruptions in the incretin system are an early key player in the pathogenesis of diabetes.

- The incretin system is further 'influenced' by the degree and duration of dysglycaemia.

- South Asians are at high risk of developing type 2 diabetes. There is evidence to suggest early signs of dysglycaemia including insulin resistance, well before the onset of overt type 2 diabetes, amongst South Asians. The literature also suggests that most South Asians are diagnosed with diabetes much later and with advanced disease compared to Caucasians.

For the above reasons, our study was designed to include South Asians with normal glucose tolerance.

The study was originally designed to investigate three key aspects of the incretin system

- Firstly, we aimed to make quantitative comparisons of the incretin effect between both groups

- Secondly, we aimed to compare endogenous incretin responses to differing meal compositions

- Thirdly we aimed to compare insulinotropic responses to intravenous infusions of GLP-1 and GIP amongst both groups.

However due to time restrictions and challenges in procuring GMP- certified GLP-1 and GIP hormones, the study was limited to the above two aims only.

Aims

Our study was aimed to answer the following key questions:

Compared to normal glucose tolerant Caucasians with similar age and BMI,

1. Is the incretin effect significantly different in South Asians?
2. Do endogenous incretin responses to meals differ in South Asians?

1.18.1.1 Experiments

1) Measure quantitative differences in the incretin effect
2) Compare postprandial incretin responses to isocaloric carbohydrate rich, fat rich and mixed liquid meals

1.19 Hypothesis

We hypothesised that the incretin system in glucose tolerant South Asians is different from Caucasians with similar age and BMI.

This may potentially be due to a difference in the quantitative incretin effect, endogenous postprandial responses of individual incretin hormones, susceptibility to DPP-4 inactivation and/or insulinotropic or alpha cell responses to circulating incretin hormones.

Chapter 3

Subjects, materials and methods

Subjects, materials and methods

1.20 Ethical Approval

The study was sponsored and approved by The Royal Liverpool & Broadgreen University Hospitals NHS Trust, United Kingdom.

Ethical approval was obtained from the National Research Ethics Service (NRES Committee North West - Greater Manchester East) [(REC reference 11/NW/) (REC approval letter Appendix).

All experiments were conducted on the Clinical Research Facility at the sponsor institution, in accordance with the principles of the Declaration of Helsinki.

1.21 Subjects

Twelve South Asians and fifteen Caucasian subjects were recruited within the study. Not all participants were included in both parts of the study (incretin effect and meal responses) due to various reasons including unavailability of individual subjects or due to difficult venous access for repeated blood sampling.
Both groups included subjects with similar BMI and age ranges. I did not however match participants in both groups for age and BMI.

Identification

Participants were recruited from the general public. Study adverts were displayed in communal areas within the hospital. In addition, the study was advertised online via the university of Liverpool website as well as the sponsoring trust's (the Royal Liverpool and Broadgreen University hospitals NHS trust) internal website.

Consent process

Initial information regarding the study was provided as outlined in Participant Information Sheet part 1.

Potential participants were then screened for eligibility and given further information by the principle investigator (Participant Information Sheet part 2). Potential participants were then offered at least one week to decide prior to giving consent. Written consent was obtained after this one-week period.

Inclusion/Exclusion criteria

1.21.1.1 Inclusion criteria

Matched groups (for age and gender) of South Asians and Caucasians were recruited.

1.21.1.2 Age Groups

21 -75 years of age

1.21.1.3 Gender

Both males and females would be recruited for the study.

1.21.1.4 Glycaemic status

Only those with normal glucose tolerance were recruited. Normal glucose tolerance was established following response to a 75 g. oral glucose tolerance test (OGTT).

1.21.1.5 Exclusion Criteria

Diabetes including both type 1 or type 2 diabetes
Anaemia (Hb<12 gm./dL)
Deranged liver function tests (ALT, AST, ALP or GGT greater than 2 times upper limit of normal)
Impaired renal function test (eGFR<60 mls/min/1.73m^2)
Previous pancreatitis
Previous gastrointestinal surgery
Unable to comply
Unable to complete tests
Any long-term disease including diabetes, hypertension and dyslipidaemia
On long-term oral steroid treatment for any other reasons.
Active malignancy
Ongoing participation in any other studies or trials.
Any other racial descent besides white European (Caucasian) or South Asian

1.22 Screening visit

Oral glucose tolerance test

As part of screening, all potential study participants underwent an oral glucose tolerance test. This was done following an overnight fast (10 hours). Samples for blood glucose were drawn at 0 minutes and 120 minutes. All participants consumed 480 mls. of Lucozade^{R} (equivalent to 75 g. of carbohydrates) after the 0-minute sample.

Confirmation of normal glucose tolerance was made using the WHO criteria.

Other tests

At screening, in addition to the OGTT (as described above), subjects also had additional routine tests including a full blood count, liver function tests, renal function tests, glycosylated hemoglobin and a fasting lipid profile.

One of the potential participants failed at screening as confirmed to have impaired glucose tolerance.

1.23 Study Protocol

After screening, the study was divided into 2 parts based on experiments.
Ideally all recruited participants were offered to go through both parts of the study. However, some participants were involved in only one part of the study due to either self-exclusion (time commitment) or due to access (difficult cannulation) issues.

Study design

For all experiments, subjects were required to attend after an overnight fast. Also, all subjects were advised to follow their usual diet and lifestyle for 48-hrs. prior to the experiment.

1.23.1.1 Experiment 1- Quantitative measurement of incretin effect

This part of the study involved two study experiments performed in a fixed order.
The experiments also involved isoglycaemic clamp studies. As these are not commonly used methods, I visited one of the leading groups in Denmark (Knop & Vilsboll, Gentofte Hospital), to learn techniques of performing isoglycaemic clamp studies.
I successfully secured a laboratory visit grant from the Society of Endocrinology (16th -18th November 2011) for assistance to visit the unit in Denmark.
As part of my visit, I observed PhD students performing isoglycaemic clamps studies on humans.
Subsequently I was awarded the Samuel Leonard Fellowship grant by the Royal College of Physicians. The grant supported me to visit Professor Jens Holst's laboratory (Panum Institute, Copenhagen) in Denmark, for a week (February 2013). During this period, I learnt about the various assay methods used in measuring GLP-1, GIP and glucagon within the laboratory.
During my visit, I also observed incretin studies being performed on vagotomised pigs and rats.

Day 1- OGTT
This involved a 4-hr. sampling following a 50-gram oral glucose load. In addition, two basal samples over a 15-minute period, prior to the glucose challenge, were also drawn. Subsequently blood samples were drawn every 5 minutes. Cannulae were inserted five minutes before sampling and then removed after drawing the final sample.
Subjects consumed a standard meal of their choice at the end of the test.

Day 2- IGII
This involved an isoglycaemic intravenous glucose infusion (IGII) study to replicate the glucose values achieved during each subject's 50-gram OGTT. Samples were drawn as per same schedule as in experiment 1.
Subjects consumed a stand meal of their choice at the end of the test.

1.23.1.2 Experiment 2- Comparing postprandial incretin responses to three different (carbohydrate rich, fat rich and mixed) liquid, isocaloric meals.

This part of the study included 3 experiment per subject performed at least forty-eight (48) hours apart.

Liquid Meal compositions:
Firstly, liquid meals were preferred over solid meals for our study, to circumvent the issue of different gastric emptying rates, a potential confounding factor. Liquid meals minimise potential variations in post-prandial hormone levels resulting due to varying gastric emptying rates (243). At the same time similar to solid meals, liquid meals are also known to elicit robust incretin hormone responses (244).

All liquid meals were also consumed with paracetamol to allow indirect measurement of gastric emptying.

1.24 Biochemistry

Blood samples were decanted into chilled tubes (Sarstedt) containing EDTA, aprotonin (500KIU/ml of blood; Nordic Pharma, Reading, United Kingdom) and valine pyrrolidide (final concentration of 0.01 mmol/L, a gift from Professor F. K. Knop) for measurement of GIP and GLP-1 levels. For measurement of glucagon, samples were distributed in chilled tubes containing EDTA and aprotonin (500 KIU/ml of blood; Nordic Pharma, Reading, United Kingdom).

For measurement of glucose, blood was distributed in tubes (Sarstedt) containing fluoride. Lastly samples were decanted in serum gel (Sarstedt) tubes for measurement of insulin, c-peptide, paracetamol and free fatty acid levels.

Serum gel tubes were allowed to stand for 20 minutes at room temperature for coagulation and all EDTA tubes were kept on ice till centrifugation.

All sample tubes were centrifuged at 4000 rpm for 10 minutes at 4 degrees. All samples were stored at -80 degrees until analysis.

Methods of individual analysis are described below

Glucose

Plasma glucose was measured using UV testing and the enzymatic reference method with hexokinase using Glucose HK Gen.3 (cobas®, Roche Diagnostics).

Insulin

Insulin was measured using an automated electrochemiluminescence immunoassay (ECLIA) based on the sandwich principle (cobas®, Roche Diagnostics). The lower detection limit for insulin is 1.39 pmol/L (0.2 milli unit/ml).

C peptide

Similar to insulin, C-peptide was also measured using an automated electrochemiluminescence immunoassay (ECLIA) based on the sandwich principle using a ROCHE cobas analyser. The lower detection limit for c-peptide is 0.003 nmol/L. The cross-reactivity with intact and split proinsulin in the C-peptide assay was insignificant.

Non-esterified free fatty acids (NEFA)

NEFA was measured using an automated COBAS analyser based on the enzymatic colorimetric test.

Paracetamol (acetaminophen)

Paracetamol (acetaminophen) was measured using a colorimetric assay with an automated (ROCHE cobas) analyser. The lower detection limit for the assay is 7.94 µmol/L.

Paracetamol absorption test:
This was used as an indirect measure of gastric emptying. The basis of the test is the fact that paracetamol is absorbed from small intestines only. Therefore the time taken for achieving peak paracetamol levels in blood after a single administration, would be indicative of gastric emptying time (245). This is a commonly used test in various incretin studies as a non-invasive surrogate marker for estimating gastric emptying time.

Triglycerides

Triglycerides were measured using an automated COBAS analyser based on the enzymatic colorimetric test.

Professor Jens Holst's group at the Panum institute, Copenhagen, Denmark, undertook the analyses for glucagon, total GLP-1 and GIP. Further details are as follows

Glucagon

The glucagon assay was directed against the COOH terminus of the glucagon molecule (antibody code no. 4305) and, therefore, measures glucagon of pancreatic origin (246) only. Neither glicentin or oxyntomodulin cross-react, but proglucagon(1-61), which is mainly formed in the pancreas does react fully in this assay (247, 248).

GLP-1

Plasma samples were assayed for total GLP-1 immunoreactivity as previously described (144) using an antiserum (no. 89390) which is specific for the COOH- terminus of the GLP-1 molecule and reacts equally with intact GLP-1 and the primary(NH_2 -terminally truncated) metabolite with a detection limit of 1.0 pmol/L.

Total GIP

Total GIP was measured based on radioimmunoassay using ethanol for extraction, as previously described (249, 250). Total GIP was measured using the COOH- terminally directed antiserum R65, which reacts fully with intact GIP and the NH_2-terminally truncated metabolite. The detection limit of the assay is 2.0 pmol/L.

1.25 Visual analogue scale

For part 2 (liquid meals) of the study, data were collected on satiety and hunger using a visual analogue scale. This was done at regular intervals of each experiment.

1.26 Devices

Body impedance analyser

A standard bioelectrical impedance analyser (BIA) was used for measurement of weight, estimation of body mass index and total body fat percentage including visceral adiposity. We used the Tanita Body Composition Analyser (SC331S). This device estimates body composition by measuring bioelectrical impedance.

Principle: Bioimpedance analysis is a non-invasive method for accurate analysis of body composition using electrical resistance (impedance) of various tissues of the body. This is based on a two compartment body composition model(2C model dividing body weight into fat mass and fat free mass) which assumes known and constant proportions of fat free mass as water, protein and mineral (251). Fat within the body allows almost no electricity to pass through while electricity passes through easily through water, much of which is found in muscles. The degree of difficulty with which electricity passes through a substance is known as the electrical resistance, and the percentage of fat and other body constituents can be inferred from measurements of this resistance. An estimate of total body water is acquired, and this helps to calculate the total body fat free mass, based on the assumption that 73% of the body's fat free mass is water. While bioimpedance analysis would correlate well with estimation of abdominal adipose tissue, it is less reliable for visceral adiposity measurements (252). However, electrode application on different areas or using multifrequency impedance analysers, compared to leg-to-leg mode has been shown to improve accuracy of estimation of visceral adiposity (253). Validity of bioimpedance analysis is also influenced by sex, age, disease state and ethnicity (254).

The Tanita Body Composition Analyzer measures body composition using a constant current source with a high frequency (50kHz, 90μA) based on foot-foot measurements. The 4 electrodes are positioned so that electric current is supplied from the electrodes to the tips of the toes of both feet, and voltage is measured on the heel of both feet.

The current flows into the upper limbs or lower limbs, depending on the body part(s) to be measured. This also measures a visceral rating as a marker of visceral adiposity.

Visceral Fat Rating

This feature indicates the rating of visceral fat in the body.
The Tanita Body Composition Analyzer provided with a visceral fat rating
from 1 – 59.

-Rating from 1 to 12
Indicates healthy level of visceral fat.

- Rating from 13 – 59
Indicates excess level of visceral fat.

YSI glucose analyser

We used a YSI 2300 STAT (Yellow Springs, OH, USA) glucose analyser for near patient glucose testing during the isoglycaemic infusion clamp studies. Plasma glucose concentrations were measured by the analyser using the glucose oxidase method.

The analyser was loaned by Professor Cuthbertson, Aintree University Hospitals, Liverpool.

1.27 Statistics

Data analysis

Data were analysed using the PRISM software.
All results are expressed as means ± SEM unless otherwise stated.
Area under curve (AUC) was calculated using the trapezoidal rule. Incremental AUC were obtained by deducting the area below baseline from total AUC. Normally distributed data were compared using paired two-tailed t tests. For data not following a normal distribution, significance of differences was tested using a Wilcoxon test for paired comparisons and Mann Whitney U test for unpaired comparisons.
A p value of <0.05 was considered statistically significant.

Sample size

The study sample size was determined using a significance level of 5%, a power of 80% and a minimal relevant difference of 15% between groups. The standard deviation (SD) of the primary endpoint (incretin effect) was based on previous studies (255). We recruited a minimum of 12 participants in each group per study, allowing for a 10% dropout rate and ensuring a minimum of 80% power despite this dropout rate.

1.28 Indices measured

Assessment of insulin secretion

1.28.1.1 - Insulin secretion rate (ISR)

ISR values were calculated by deconvolution of measured C-peptide concentrations and application of population-based parameters for C-peptide kinetics as described previously (256). ISR is expressed as picomoles insulin secreted per minute per kilogram body weight.

1.28.1.2 - Insulinogenic index (II)

It's a measure of beta cell sensitivity based on the principle that beta cells respond proportional to a rising blood glucose concentration(257). A ratio relating to enhancement of circulating insulin to magnitude of corresponding glycaemic stimulus, was used to compare the secretory capacities of respective groups. It does not take body weight in the equation.

II was calculated following all three meals, as the ratio of the increment in plasma insulin concentration to the increment in the plasma glucose concentration from time 0 to time 30 min only.

Assessment of insulin sensitivity and resistance

1.28.1.3 - HOMA (IR)

Homeostatic model assessment (HOMA) is a method for assessing beta cell function and insulin resistance from basal (fasting) and insulin or C-peptide concentrations (258). For the purpose of our study, we only used the HOMA2 model for measurement of insulin resistance between both groups.

1.28.1.4 - Disposition Index (DI)

The relationship between insulin sensitivity and insulin secretion is thought to be approximately hyperbolic so that the product of the two variables is constant for individuals with the same degree of glucose tolerance(259). This constant was first termed the 'disposition factor' (260), but is now commonly known as the disposition index. Hence disposition index is a measure of beta cell response relative to the insulin resistance.

Originally the disposition index was calculated from the insulin sensitivity and acute insulin secretion drawn from the frequently sampled intravenous glucose tolerance test (FSIGT). As a result, the disposition index is a measure of whole-body insulin sensitivity.

For the purpose of our study, the disposition index was calculated as a product of the insulinogenic index and the Matsuda index.

1.28.1.5 - Matsuda Index

This is an index of whole body insulin sensitivity that represents a composite of hepatic and peripheral tissues (261). This is calculated as 10000/square root of (fasting glucose X fasting insulin) x (mean glucose X mean insulin) including all time points till blood glucose returned to basal readings.

For the purpose of our study, Matsuda index was calculated using the web calculator (**http://mmatsuda.diabetes-smc.jp/MIndex.html**) based on time points from 0 to 120 minutes.

From: **Julie Cragg** julie.cragg@endocrinology.org
Subject: RE: Lab Visit grant
Date: 11 November 2011 14:16
To: Rupa.Ahluwalia@liverpool.ac.uk
Cc: Ann Lloyd ann.lloyd@endocrinology.org

Dear Rupa
Thank you for your application for a lab visit grant which has been approved by the Grants Panel. I will leave the rest of the process to my colleague Ann, whom I have copied in but, in view of the fact that the visit is due to start next week, I thought you would like to know the outcome. Ann will be back in the office next Tuesday and will get in touch.

Best wishes
Julie

Julie Cragg
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From: Ahluwalia, Rupa [mailto:Rupa.Ahluwalia@liverpool.ac.uk]
Posted At: 10 November 2011 10:59
Posted To: Grants
Conversation: Lab Visit grant
Subject: Lab Visit grant

Dear Ann,

Please find attached my lab visit grant application.As im planning to visit next week, I would be grateful for all your help in expediting the application.
The email from the host researcher(Dr Knop) would be sent shortly.
Also I haven't mentioned the institution account details yet as need to work out the one which would be least cumbersome for transactions.I hope this would not affect the application process.

1

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Chapter 4

Comparing incretin responses following isocaloric liquid meals of varying compositions

Experiment three, four and five

Introduction

Incretins are intestinal hormones secreted from the enteroendocrine cells, released within minutes of food intake (120). Both GIP and GLP-1 act as incretin hormones that augment glucose stimulated insulin secretion (262). The insulinotropic effects of GIP and GLP-1 are additive and together fully explain the incretin effect (263).
In healthy subjects, the incretin hormones may account for almost 70% of the postprandial insulin response and thereby indirectly facilitate uptake of glucose in muscle, liver and adipose tissues (126). Incretin hormones are now known to influence a considerable part of the postprandial insulin response (128).

Both GLP-1 and GIP are known to be secreted in to the hepatic portal venous system by specific enteroendocrine cells located in the intestinal mucosa, in response to food ingestion (264). The GLP-1 and GIP response to meal stimuli is influenced by meal composition, size as well as endogenous insulin status.
These two incretin hormones are also secreted during mixed nutrient meals when insulin secretion is augmented beyond the effects of hyperglycaemia alone (265). Hence meal stimulated secretion of incretin hormones and insulin provides important insights into glucose homeostasis and energy balance (266).
There are various factors influencing the postprandial GLP-1 and GIP responses as discussed below.

1.29 Meal compositions

Macronutrients have the capacity to stimulate GLP-1 and GIP release, however with differential potency.

Most of the earlier studies have focused only on the effects of glucose (carbohydrates) in stimulating GLP-1 and GIP secretion.
Vollmer et al (141) demonstrated that GIP responses were higher following a mixed meal and not during OGTT, in both healthy subjects and those with type 2 diabetes. Seventeen patients with type 2 diabetes, 17 subjects with impaired glucose tolerance and 14 control subjects with normal glucose tolerance were studied following an oral glucose tolerance test and a mixed meal (820 kcal) over 240 minutes on two separate occasions. The initial GIP response was exaggerated compared to control subjects after a mixed meal ($p<0.001$) but not after an oral glucose tolerance test ($p=0.98$). GIP responses were 186±17% higher after mixed meal ingestion than following oral glucose load ($p<0.0001$). However, GLP-1 responses were similar following both stimuli. There was also a negative association between fasting glucagon and integrated FFA and subsequent GLP-1 concentration. However fasting glucagon, integrated FFA and female sex were positively related to GLP-1 concentrations (Vollmer Diabetes 2008)(141).

A small study of healthy individuals has also shown that the plasma GIP concentrations paralleled the gastric emptying of fat and protein (267). In this very early study, 5 healthy volunteers with a multi-lumen duodenal tube ingested a mixed meal with specific markers for aqueous phase, liquid fat and solid protein phase. The study demonstrated that the plasma insulin concentration showed no correlation to the rate of emptying of fat and protein and to the plasma GIP level. On the other hand, the time course of plasma GIP concentration did parallel the gastric emptying of fat and protein ($r=0.763$-0.834; $p<0.01$-0.05). However, the observed threshold for plasma glucose at which GIP would exert an incretin effect was only reached at one time point which was 30 min after ingestion of the meal. Hence the investigators concluded that in humans, their findings did not support an important physiological role of GIP as an insulinotropic hormone after ingestion of mixed meals.

While a few studies have looked at the effect of fat and protein ingestion on incretin hormone secretion, the nutrient stimuli used in these studies were much greater (132, 264).

More recently a further study by Carrel et al, have looked at incretin responses post fat and protein ingestion using much smaller stimuli i.e. similar to calories consumed during normal eating (268). Using a hyperglycaemic clamp along with oral nutrients, this study assessed the incretin effects elicited by mixed meals as well as by its fat and protein components alone. Eight healthy men were studied on four separate days under hyperglycaemic clamp conditions (glucose 8 mM), in combination with a mixed (whole sandwich with dried meat(10 g), butter(20 g) and white bread(120 g): 63.4 g CHO, 18 g Fat, 14.9 g protein), protein rich (dried meat(40 g): 15.4 g Protein) or fat rich (butter(20 g): 16.4 g fat) meal during three out of the four days. The study showed that ingestion of sandwich (mostly carbohydrate) and butter alone (lipid) but not that of dried meat (mostly protein), resulted in increased glucose induced insulin production. The group also demonstrated that the lipid component made a significant contribution to the incretin effect of a mixed meal while it's protein component made little contribution (268).

Carr et al(2008) (269) looked at whether incretin hormones contributed to the islet hormone secretion after non-carbohydrate macronutrient ingestion. The investigators studied incretin and islet hormone responses in 12 healthy individuals following ingestion of pure fat (oleic acid only 0.88 g/kg) or protein (milk and egg protein; 2 g/kg) over a 5-hour period. Plain water ingestion served as a control. The group noted that GLP-1 responses were similar after protein and fat ingestion whereas the early (30 min) GIP response was higher after protein than after fat ingestion ($p<0.001$). Protein ingestion was associated with seven-fold higher insulin and glucagon responses than fat ingestion (both $p<0.001$).

Following protein ingestion, the early GIP but not GLP-1 responses correlated to insulin (r^2 =0.86:p=0.0002) but not glucagon responses. However, following fat ingestion, both GIP and GLP-1 did not correlate to islet hormone responses. As a result Carr et al (269) concluded that early GIP secretion after protein ingestion might be of primary importance to islet hormone responses.

Whereas fat preferentially stimulates GIP secretion, carbohydrates have been demonstrated to primarily stimulate GLP-1 secretion in humans (132). In this randomised crossover study, 18 subjects with type 2 diabetes and 6 healthy volunteers underwent 3 four-hour meal tolerance tests (small CHO rich, large CHO rich and fat rich meals). Besides demonstrating differences in insulin and incretin hormone responses as a result of differing meal sizes, the study also showed that not only did fat preferentially stimulate GIP release, GIP levels were continuing to rise even 2 hours following the meal.

The mechanism of incretin hormone stimulation also varies for individual macronutrients. Carbohydrates, for example, stimulate incretin secretion through a number of interrelated mechanisms that are likely to include the sodium-glucose cotransporter-1 (SGLT-1) and intestinal "sweet taste" receptors (120).

At the same time the interplay between individual macronutrient ingestion and DPP-4 activity is not fully understood.

As we know DPP-4 activity influences the amount of circulating 'active' incretin hormones. By measuring total GLP-1 and GIP levels, we are estimating secretory incretin hormone responses only and not the active hormone levels.

Gunnarson et al (2006) have shown reduced intestinal DPP-4 activity following protein but not carbohydrate ingestion amongst mice. Therefore, while we measure GLP-1 and GIP to compare incretin hormone responses, what we don't know is the effects of the individual macronutrients on DPP-4 activity and consequent active incretin hormone levels. Carr et al (269) studied effects of protein and fat ingestion on islet hormone levels measuring both active and total GLP-1 and GIP levels. Their study also showed comparable DPP-4 activity following protein or fat ingestion.

The group also showed that like fat, protein ingestion also has the ability to stimulate GIP secretion (269). This would be in keeping with previous study whereby intraduodenal infusion of amino acids stimulated GIP secretion (270).

Interestingly Carr et al (269) also noted that protein ingestion resulted in a higher early GIP response in comparison to fat. However late GIP responses were similar following fat and protein ingestion. On the other hand, both macronutrients appear to have a similar response in terms of GLP-1 secretion. It was also noted that the effects

on GLP-1 and GIP following ingestion of individual macronutrient were sustained for some time, as the levels remain raised even 5 hours later (269). The counterargument to the findings of this specific study is based on the fact that meal stimuli used for these experiments was much higher than found in a 'usual diet'.

1.30 Meal size

Magnitude of GLP-1 and GIP responses is proportionate to the caloric content of the meal (132). The same group also showed that larger carbohydrate load results in a larger GLP-1 release regardless of the glycaemic status.

Vilsboll et al (131) studied meal induced incretin responses in state of health (n=8), obesity (n=8), type 1(n=8) and type 2 diabetes (n=8). Incretin and insulin responses were measured until 180 minutes following ingestion of the breakfast meal. The incretin hormone responses were significantly higher in all groups following a large meal (520 kcal) compared to a small meal (260 kcal). Early total GLP-1 responses (tAUC 0-30) were similar in all 4 groups during both meals, while the late responses (tAUC 30-180) were significantly higher during large compared to small meal tests. Decreased GLP-1 responses were noted amongst patients with type 2 diabetes in comparison to obese healthy subjects ($p<0.02$, 90 min). Early intact GLP-1 responses increased in the two patient groups during a large meal compared to a small meal. However, the early intact GLP-1 responses were the same during both meal tests in the two groups of healthy subjects. The late intact GLP-1 responses tended to increase in all groups during a large meal but significantly so in the two groups of diabetic subjects. The incremental intact GLP-1 to the small meal (AUC 0-30 min) was lower in subjects with type 2 diabetes compared to healthy subjects ($p=0.04$)

Both type 1 and type 2 diabetes subjects had normal GIP responses in comparison to healthy subjects. The study also demonstrated increased beta cell sensitivity to glucose in subjects with type 2 diabetes (29%) and obese healthy subjects (22%) during a large meal compared to a small meal. This change in beta cell sensitivity was seen despite only a minor insignificant increase in plasma glucose levels. The authors attributed this increased beta cell sensitivity to increased GLP-1 secretion (131).

1.31 Body weight

In addition, incretin responses are also dependent on body weight.
Vilsboll et al (131) as mentioned in the previous section, have also demonstrated effects of obesity on postprandial incretin responses. In comparison to lean healthy controls, subjects with obesity had increased fasting concentrations of GIP and an early-enhanced postprandial GIP response, while the GLP-1 responses were similar in both groups. The same group have also shown that GLP-1 elimination is the same in obese type 2 diabetic subjects and matched healthy subjects (131).

Ranganath et al (271) examined the effects of obesity on incretin hormone responses. Subjects include obese women (mean BMI 40.1) in comparison to matched controls (mean BMI 21.3). In a study involving 6 subjects with matched controls, all received, in a random order, equicaloric (340 kcal) oral load of either carbohydrate or fat. An intravenous bolus of heparin 10,000 units was given 2 hours after the carbohydrate/fat loads to assess lipoprotein lipase activity. The mean fasting glucose prior to carbohydrate loading was lower in obese subjects ($p<0.05$) with no similar differences noted prior to fat loading. Incremental GLP-1 responses (0-180 min) post carbohydrate loading was considerably lower in the obese (iAUC (SD) 4.8(1.5) vs. 0.3(0.4) picomol/min for lean and obese subjects respectively; $p<0.01$) subjects. There was decline in plasma concentrations of GLP-1 at 150 minutes, 30 minutes after the heparin infusion in both lean and obese subjects, when compared to the 120 minutes. Plasma GIP levels were similar in lean and obese subjects after both carbohydrate and fat meals (iAUC(SD) for carbohydrate meals was 4(5) and 39(5.3) picomol/min respectively in lean and obese; iAUC(SD) for fat meals was 46(7.4) and 44(4.2) picomol/min respectively in lean and obese).The investigators demonstrated attenuated GLP-1 responses after carbohydrate but not fat loading amongst obese subjects. There were no differences in the measured responses amongst lean and obese subjects following a fat stimulus. Also, the GIP response following a carbohydrate stimulus was similar in both groups. After a carbohydrate stimulus, obese subjects also showed less efficient glucose disposal and higher plasma insulin concentrations. The investigators concluded that lower GLP-1 concentrations following

carbohydrate ingestion in obese subjects may have a role to play in peripheral insulin resistance which is characteristic of obesity(271).

A further study by Toft-Nielsen et al (169) looked at determinants of the impaired GLP-1 response in subjects with type 2 diabetes. The authors studied responses to a mixed meal amongst 54 subjects with type 2 diabetes, 33 matched controls with normal glucose tolerance and 15 unmatched healthy controls. The post meal GLP-1 response (total AUC; 0-240 min) was significantly reduced in those with type 2 diabetes (2482± 145 vs. 3101 ± 198 picomol/l; p=0.024). This is discussed at length below. The study did show a negative association between BMI and GLP-1 responses.

Similarly Muscelli et al (166) also demonstrated an inverse relationship between the incretin effect and both BMI as well as glucose tolerance (2 hr. plasma glucose level) in an independent, additive manner. Findings are discussed in detail in a subsequent chapter.

1.32 Insulin resistance

The influence of insulin on the incretin hormone secretion is less well established. There are some earlier studies supporting the hypothesis that insulin resistance may be contributory to the impaired incretin hormones responses in humans.

Insulin appears to have a negative feedback effect on the enteroinsular axis (272). A previous animal study supporting a similar concept of inhibitory impact of insulin on incretin hormones, demonstrated a reduced GIP response to intraduodenal administration of glucose, following exogenous insulin (273).

In the context of insulin resistance, there is both impaired and enhanced postprandial incretin hormone responses reported in humans.

Insulin resistance has also been demonstrated to be associated with impaired postprandial incretin hormone responses (167). Rask et al (167) showed an inverse relationship between insulin sensitivity and GLP-1 responses following a mixed meal (GLP-1 levels in first 15 minutes were significantly lower in the most insulin resistant tertile compared with the most insulin sensitive tertile). In addition, in the first hour, the AUC of GLP-1 correlated significantly with insulin sensitivity. The GIP responses at 60 minutes were significantly lower in the insulin resistant compared to the insulin sensitive group (plasma GIP $p<0.01$; tAUC GIP $p<0.05$). However basal levels of GIP and GLP-1 were similar. Overall this study showed an early attenuation of the GLP-1 response and a diminished GIP secretion later in the postprandial period.

A much more recent study by Hansen et al, compared incretin responses to liquid meals, before and after an intervention inducing insulin resistance using high calorie diet, relative physical inactivity and administration of prednisolone (274). As a result of the intervention, the study showed no difference in postprandial GLP-1 levels but in exaggerated postprandial GIP and glucagon responses. The findings by Hansen et al varies from those of Rask et al (167) . However, there are also key differences in both studies as one used liquid while the other uses solid meals. Also subjects in the study by Hansen et al (274) have short-term insulin resistant state with mild glucose intolerance, induced by interventions including glucocorticoids. In contrast Rask et al (167) studied subjects with endogenous insulin resistance but normal glucose tolerance.

1.33 Post prandial incretins and type 2 diabetes

GLP-1 responses:

There have been other studies demonstrating impaired meal induced GLP-1 responses in the context of insulin resistance in subjects with type 2 diabetes (131, 169, 275). However once again insulin resistance in these subjects was longstanding with glucose intolerance, both of which can themselves independently have an impact on the incretin hormone responses. Overall postprandial GLP-1 responses in subjects with type 2 diabetes have been shown to be decreased or even normal (131, 141, 275). Vilsboll et al (275) also noted that the GLP-1 profile, following a mixed meal, was characterised by a small early rise (30-45 minutes) and a significantly reduced late phase response (75-150 minutes).

This evidence to some extent supported the hypothesis that an impaired GLP-1 response in type 2 diabetes contributed to the overall inappropriate insulin secretion (275). Vilsboll et al (131) have also shown that the GLP-1 elimination is similar in obese subjects with type 2 diabetes compared to healthy subjects. Hence the impaired incretin hormone response after ingestion of a standard meal in type 2 diabetes has been attributed to decreased secretion of GLP-1.

Interestingly, no improvement in postprandial GLP-1 secretion has been noted despite restoration of normal glucose tolerance in subjects with type 2 diabetes (276). Postprandial (mixed meal) responses amongst nine subjects with type 2 diabetes were compared before and after change in glycaemic control (Hba1c of 8 vs. 6.6%). While there were no changes in the actual postprandial incretin hormone levels before and after the intervention, the beta cell responsiveness was significantly improved following near normalisation of glucose levels. Also, there was evidence of significant improvement in the insulinogenic index.

The same group also studied beta cell responsiveness to GLP-1 and GIP, before and after near normalisation of glucose levels, in type 2 diabetes (177). In this study, eight obese individuals with type 2 diabetes (poor glycaemic control) were studied before and after near normalisation of blood glucose levels. Responses during 3 different hyperglycaemic clamp studies (saline, GLP-1 and GIP) were compared before

and after intervention. The study demonstrated improvement in the late phase (10-120 min) C-peptide responses following both GIP and GLP-1 infusions.

Lugari et al has studied effects of meal ingestion on GLP-1 secretion in subjects with type 1 and type 2 diabetes (212). The group studied 16 subjects with type 1 diabetes, 14 with type 2 diabetes and 10 matched controls. All subjects consumed a mixed breakfast of 230 kcal with samples drawn for 3 hours post ingestion. The study showed a significant increase in postprandial GLP-1 levels in healthy controls but not in subjects with type 1 or type 2 diabetes. On the other hand, GLP-1 levels were reduced in type 2 diabetes subjects.

GIP responses:

It has been speculated that in glucose tolerant, healthy weight individuals, there is a negative feedback between insulin secretion and GIP response (277). Hence it has been postulated that the exaggerated postprandial GIP response in obese and/or subjects with type 2 diabetes may occur as a result of a defective negative feedback mechanism possibly due to down regulation of insulin receptors on GIP secreting K cells, as proposed by Creutzfeldt et al (277). Evidence supporting this hypothesis has been demonstrated in studies amongst obese subjects (278) as well as those with type 2 diabetes (279). Exaggerated GIP responses have also been noted amongst healthy offsprings of individuals with type 2 diabetes (280). However fasting and postprandial GIP responses were similar amongst subjects with type 2 diabetes compared to healthy controls (275). Vilsboll et al (2003) (131) showed no difference in postprandial GIP responses in type 2 diabetes subjects when compared to healthy subjects, following both large (520 kcal) and small sized (260 kcal) meals.

1.34 Gastric emptying

Gastric emptying is also known to play a key role in postprandial glycaemia (281). There is significant inter individual variation in gastric emptying in health and much more in the context of diabetes where gastric emptying may be delayed or even accelerated. It has previously been reported that gastric emptying accounts for 35% of the variance in glycaemic responses (both peak and total AUC) to oral glucose and/or carbohydrate-containing meals in normal glucose tolerant individuals (282) as well as in those with type 2 diabetes (283).
Altered gastric emptying influences absorption rate in the proximal intestine which in turn alters the transit time of the remaining food i.e. stimuli reaching the distal intestine where GLP-1 producing L cells are most abundant, ultimately impacting postprandial incretin responses. Therefore, increased proximal absorption may result in reduced GLP-1 secretion. This has been demonstrated in a study involving liquid meals in obese subjects with high proximal absorption rates (284). Vilsboll et al have proposed this as one of the possible explanations (131) for reduced GLP- secretion in obesity, as also reported by various groups (169, 271, 285).

In addition, gastric emptying is also influenced by plasma glucose levels. Acute hyperglycaemia is known to delay gastric emptying while hypoglycaemia is known to cause accelerated gastric emptying (281, 286). Blood glucose levels of even 8 mmol/mol are known to delay gastric emptying (287).

Meal compositions itself can influence gastric emptying. Fat, in comparison to protein and carbohydrates, is known to slow gastric emptying. Therefore, the influence of individual macronutrients needs to be taken into account when comparing variations in incretin hormone responses.

Meals but not oral glucose tolerance tests have shown to be associated with reduced postprandial GLP-1 responses in type 2 diabetes (169, 263, 275, 276).

There is some evidence to suggest that South Asians have a varying response to meals in comparison to Caucasians. A study comparing response to biscuits and cereals in South Asians and Europeans, demonstrated that South Asians had a higher glycaemic response (288). A study amongst Chinese subjects demonstrated greater glycaemic response to various varieties of rice, in comparison to Europeans (289). While Chinese and South Asians are not in the same racial group, a study by Tey et al, comparing glycaemic responses to three different liquid meals of varying composition amongst Indians, Chines and Malay subjects showed no difference in glycaemic responses between the three different racial groups (290).

However, there is no study directly comparing incretin hormone responses following meals in South Asians and Caucasians. Study by Sleddering et al as previously discussed, demonstrated higher GLP-1 and higher insulin responses in young, healthy South Asians compared to Caucasians, following a prolonged 75-g OGTT. (196). However, what is not known is whether these differences in incretin hormone responses amongst South Asians, are also present following meal ingestion.

To the best of our knowledge, ours is the first study making contemporaneous comparisons of meal induced incretin hormone responses between South Asians and Caucasians.

1.35 STUDY DESIGN/AIM:

The aim of this study was to compare postprandial incretin hormone responses between normal glucose tolerant South Asians and Caucasians.
This study included three experiments per subject. Each experiment involved consumption of either a carbohydrate- rich, fat- rich or mixed meal. All three meals were isocaloric but varying in compositions.

The aim of this part of the study was largely twofold:

- Firstly, to compare postprandial GLP-1 and GIP responses following a liquid meal, between age and BMI matched normal glucose tolerant South Asians and Caucasians.

- Secondly the study was designed to identify differences in responses to various macronutrient stimuli between both groups.

Indication for using liquid meals

There is a complex interdependent relationship between post-prandial glycaemia, gastric emptying and the incretin axis. Incretin hormone responses are dependent on gastric emptying. Therefore, in this experiment, we studied postprandial responses following three different form of liquid meals.

Subjects

Eleven South Asian (age: 35±3.7 years; BMI: 24.7±1kg/m^2; fasting plasma glucose (FPG): 4.7 ±0.18 mM) and fifteen Caucasian subjects (age: 32±3 years; BMI: 25.1±0.9 kg/m^2; FPG: 4.6±0.10 mM) with normal glucose tolerance, were recruited.
Normal glucose tolerance was confirmed following a 75-gram oral glucose tolerance test (OGTT), as per WHO criteria, which was performed prior to recruitment. None of the subjects had anaemia (Haemoglobin <12.0 g/dL), impaired renal (eGFR<60 mls/min/1.73m^2) or liver (ALT, AST, ALP or GGT greater than 2 times upper limit of normal) function tests or proteinuria.
All subjects gave written consent for participation after receiving oral and written study information.

Study Protocol

All subjects were studied on three different occasions at least 48 hours apart.
All subjects abstained from strenuous exercise for 48 hours prior to each experiment and followed their 'usual' diet during this period. All subjects omitted any regular medications including over the counter drugs, for 24 hours prior to each experiment.
For each occasion, subjects were studied in the morning following an overnight 10-hour fast. On arrival, all subjects were weighed (TANITA body composition analyser BC-420MA) followed by an ambulatory blood pressure measurement.
A cannula was inserted in the cubital vein for collection of arterialized blood samples. Blood samples were obtained twice before (-30 and 0 minutes) the meal ingestion. Subjects then consumed a carbohydrate-rich, mixed or fat-rich liquid meal with 1 gm. of effervescent Paracetamol over ten minutes. Samples were then drawn at 15,30,60,90, 180 and 240 minutes after the meal. The cannulated arm was kept warm using a heating pad in between samples.

Figure 0.1 Experiment sheet for meal studies

ISSA-4149 Study part 2

Meal tests- Visit ~~4(Mixed meal response)~~

Date: Fasting? Medications omitted?

BP: HR:

Weight: BMI:

% Body fat: Fat free mass:

Any change since recruitment/last visit?

Sample time points		**Glucose(YSI)**	**VAS score**	**Additional notes**
Sample due at	Real Time			
-30			**Y**	
0(before meal)			**Y**	
0 Liquid meal with 1.5 gm Paracetamol in 200 mls. water			**Y(after meal)**	
+15				
+30			**Y**	
+60			**Y**	
+90				
+120			**Y**	
+180			**Y**	
+240			**Y**	

Pink EDTA (1 tube with DA CHILLED) - 7 mls. (GIP+ GLP-1+ EXTRA(x2)),

Pink EDTA (1 tube with A CHILLED) - 2 ml (Glucagon + EXTRA); Orange heparin- 1ml (EXTRA)

White Serum (1 tube) - 4 mls (Insulin/Cpep, Paracetamol + EXTRA)

Fluoride EDTA (1 tube) - 1 ml (glucose)

D= DPP4i, A=Aprotonin (500 KIU/ml blood), Centrifuge@ 4 deg, 4000 rpmx10 mts.

Each plus denotes separate aliquot

Additional Notes:

Hunger and satiety were measured using a visual analogue scale (VAS) at 30 and 0 minutes before and soon after as well as 30,60,150 and 210 minutes after the meal.

Figure 0.2 Visual Analogue scale sample sheet

OFFICE USE ONLY:
PARTICIPANT NO: DATE:
VISIT NO: 1 2 3 (Please circle) TIME:

INSTRUCTIONS FOR PARTICIPANTS:

Please read each question and then put a mark through the line that best represents how you are feeling in relation to that particular sensation at this moment.

EXAMPLE:

How **TIRED** do you feel **at this moment?**

Not at all tired ——/—— Extremely tired

PLEASE ANSWER THE FOLLOWING QUESTIONS:

How **HUNGRY** do you feel **at this moment**?

Not at all hungry ——— Extremely hungry

How **FULL** do you feel **at this moment**?

Not at all full ——— Extremely full

How **SATISFIED** do you feel **at this moment**?

Not at all satisfied ——— Extremely satisfied

How **STRONG** is your desire to **eat at this moment**?

Not at all strong ——— Extremely strong

How **MUCH FOOD** do you feel you could eat **at this moment**?

None at all ——— A large amount

How **THIRSTY** do you feel **at this moment**?

Not at all thirsty ——— Extremely thirsty

How **NAUSEOUS** do you feel **at this moment**?

Not at all nauseous ——— Extremely nauseous

THANK YOU

Blood samples were decanted into chilled tubes (Sarstedt) containing EDTA, aprotonin (500KIU/ml of blood; Nordic Pharma, Reading, United Kingdom) and valine pyrrolidide (final concentration of 0.01 mmol/L, gift from Dr. F K Knop, Gentofte Hospital, Copenhagen, Denmark) for measurement of GIP and GLP-1 levels. For measurement of glucagon, samples were distributed in chilled tubes containing EDTA and aprotonin (500 KIU/ml of blood; Nordic Pharma, Reading, United Kingdom).

For measurement of glucose, blood was distributed in tubes (Sarstedt) containing fluoride. Lastly samples were decanted in serum gel (Sarstedt) tubes for measurement of insulin, C-peptide, paracetamol and free fatty acid levels.

Serum gel tubes were allowed to stand for 20 minutes at room temperature for coagulation and all EDTA tubes were kept on ice till centrifugation.

All sample tubes were centrifuged at 4000 rpm for 10 minutes at 4 degrees. All samples were stored at -80 degrees until analysis

Table 0.1 Composition of three different isocaloric liquid meals

	Carbohydrate-rich	Mixed	Fat-rich
Carbohydrates: kcal (total %)	419.42(83.3%)	269.34(52.8%)	124.94(24.9%)
Proteins: kcal (total %	61.44(12.2%)	38.4(7.5%)	16.3(3.2%)
Fat: kcal (total %)	22.5(4.5(%)	202.5(39.7%)	360(71.8%)
Total caloric content (kcal)	503.35	510.15	501.1
Total volume (mls)	350	350	350

1.36 Calculation and statistics

All results are expressed as means ± SEM unless otherwise stated. Area under curve (AUC) was calculated using the trapezoidal rule.

Incremental AUC were obtained by deducting the area below baseline from total AUC.

Insulin secretion rate (ISR) values were calculated by deconvolution of measured C-peptide concentrations and application of population-based parameters for C-peptide kinetics as described previously (Hovorka et al) (256). ISR is expressed as picomoles insulin secreted per minute per kilogram body weight. Matsuda index was calculated using the web calculator (**http://mmatsuda.diabetes-smc.jp/MIndex.html**) using time points from 0 to 120 minutes.

Insulinogenic index was calculated following all three meals, as the ratio of the increment in plasma insulin concentration to the increment in the plasma glucose concentration from time 0 to time 30 min.

Insulin resistance, mainly hepatic was measured using the homeostatic model assessment (HOMA-IR) as described by Matthews et al (291).

Normally distributed data were compared using paired two-tailed t tests. In case of data not following a normal distribution, significance of differences was tested using a Wilcoxon test for paired comparisons and Mann Whitney U test for unpaired comparisons. A p value of <0.05 were considered statistically significant.

1.1 Results

Anthropometry

Both groups were matched for age, gender and BMI. In addition, the waist hip ratios (WHR), visceral adiposity and body fat percentage (using BIA) were also non significantly different between both groups, at baseline (table 4.2).

Table 0.2 Baseline demographic and anthropometric data from both study groups

	South Asians	Caucasians	p
N (male/female)	6/5	7/8	NS
Age (years)	35+/- 3.7	32+/- 3	NS
BMI (kg/m^2)	24.7+/-1	25.1+/-0.9	NS
Waist Hip ratio (WHR)	0.85+/-0.02	0.85+/-0.02	NS
Fasting glucose mmol/L	4.7+/-0.18	4.6+/-0.10	NS
2-hr. glucose mmol/L	4.75+/-0.3	3.9+/-0.2	NS
eGFR mls/min/$1.73m^2$	88.36+/-1	84.4+/-1.7	NS
Fasting triglycerides (mmol/L)	1.6+/-0.4	1.0+/-0.12	NS
Systolic blood pressure (mm Hg)	119+/-5	121+/-3	NS
Diastolic blood pressure (mm Hg)	76+/-2	74+/-2	NS

Glucose, Insulin and C-Peptide

1.36.1.1 Glucose

Basal fasting glucose levels between South Asians and Caucasians were comparable during all three-meal tests (figure 4.3). In addition, except for the CHO-rich meal, glucose responses following the other two meals were comparable amongst South Asians and Caucasians ((total areas under the curve (total AUC) were also similar between South Asians and Caucasians (CHO: 1601±83 vs.1361±44 mmol/l×min, p=0.01*; MIX: 1383±58 vs.1260±35 mmol/l×min, p=0.06; FAT: 1254±39 vs.1192±28 mmol/l×min,p=0.19)) (figure 4.4). Significantly higher glucose responses post carbohydrate-rich and not mixed and fat-rich meals in South Asians , is concordant with previously reported findings of higher glycaemic response to carbohydrate containing foods in people of different ethnicities (289) (292).

1.36.1.2 Insulin and insulin secretion rate

Postprandial insulin responses (area under the curve (total AUC)) were higher in South Asians vs. Caucasians (CHO: 27,631±5901 vs. 10,352±900 mmol/l×min, p<0.0001; MIX: 15,548±3295 vs. 8,064±873 mmol/l×min, p=0.02; FAT: 7,228±1,092 vs. 4,027±438 mmol/l×min, p=0.006).

In addition, the differences in postprandial insulin responses were more pronounced in the second phase (30-240 minutes) rather than during the first phase (0-30 minutes). This pattern was consistent following all three different meal stimuli. The insulin responses also correlate with the late phase glycaemic responses where glucose levels amongst South Asians remained above baseline for a longer (non-significant) period of time.

Pre hepatic insulin secretory responses (using total AUCs) were also significantly higher in South Asians (CHO: 3,034±289 vs. 1,646±92 pmol/kg/min, p<0.0001 (figure 4.5); MIX: 1,980±217 vs. 1,250±67 pmol/kg/min, p=0.0002 (figure 4.6); FAT: 1,166±90 vs. 804±35 pmol/kg/min, p=0.0004) (figure 4.7).

1.36.1.3 C-Peptide

C-peptide levels post meals were numerically greater but not achieving statistical significance, in South Asians when compared to Caucasians (figure 4.8).
The differences in C-peptide levels were more pronounced during the late phase (after 30 minutes of stimuli/meal consumption).

Beta cell indices

1.36.1.4 Matsuda index

Measurement of both peripheral and hepatic insulin sensitivity using Matsuda index revealed reduced sensitivity amongst South Asians in comparison to Caucasians (CHO: 3.2±0.5 vs. 5.7±0.5, p=0.002; MIX: 4.2±0.8 vs.5.7±0.5, p=0.09 FAT: 5.2±0.6 vs. 8.7±0.9, p=0.009).

1.36.1.5 Insulinogenic index

Beta cell sensitivity measured using the insulinogenic index was comparable between South Asians and Caucasians following all three-meal stimuli.

1.36.1.6 HOMA- IR

Insulin resistance when measured using the HOMA index, was comparable between South Asians & Caucasians (CHO: 1.42 ±0.28 vs.0.83 ±0.10, p=0.05; MIX: 2.70±0.56 vs.1.74 ±0.27, p=0.14; FAT: 2.61±0.56 vs.1.81 ±0.26, p=0.09). However, South Asians had increased (non-significant) insulin resistance compared to Caucasians as reflected by higher HOMA-IR values. This would be concordant with observed reduced insulin sensitivity in South Asians (Matsuda index).

Incretin hormone responses

1.36.1.7 Total GLP-1 & GIP

Basal:

Fasting total GLP-1 were higher amongst South Asians in comparison to Caucasians (Total GLP-1: 12.3±0.52 vs. 11.5±0.49 pmol/L; p=0.01). However, fasting total GIP levels were comparable between both groups (Total GIP: 8 (6,12) vs. 7 (6,9); p=0.02).

Postprandial:

Total GLP-1

Post meals GLP-1 responses were comparable between South Asians and Caucasians (figure 4.9).

Overall the postprandial GLP-1 responses amongst South Asians were lower than Caucasians, however these differences were not significant (table 4.3).

Total GIP

Following each liquid meal, the total GIP levels were similar between South Asians and Caucasians (table 4.3). However, in the late (second) phase (30-240 min), GIP levels were higher (non-significant) in South Asians (figure 4.10).

Table 0.3 Total GLP-1 and GIP levels (total AUC) post carbohydrate rich, mixed and fat-rich meals

	South Asians	Caucasians	p
Carb-rich meal			
Total GLP-1	2880 ± 180	2886 ± 252	0.98
(pmol/L x min)	9078 ± 598	8462 ± 488	0.42
Total GIP			
(pmol/L x min)			
Mixed meal			
Total GLP-1	2640(2513,3375)	3105(2284,3949)	0.88
(pmol/L x min)	10438 ± 797	9450 ± 668	0.35
Total GIP			
(pmol/L x min)			
Fat-rich meal			
Total GLP-1	2786 ± 184	3011 ± 220	0.46
(pmol/L x min)	11389 ± 784	10628 ± 1059	0.59
Total GIP			
(pmol/L x min)			

Glucagon

Fasting glucagon levels were similar between both groups.

Nadir glucagon levels were lower in South Asians following all three meals (figure 4.11). South Asians also demonstrated a comparatively slower rise in glucagon levels following carbohydrate-rich and mixed meals (figure 4.11). Integrated glucagon responses post meals were however comparable (table 4.4) between both groups.

Table 0.4 Glucagon levels post carbohydrate-rice, mixed and fat-rich meals

	South Asians	Caucasians	p
Carb-rich meal (pmol/L x min)	1817(1226,1669)	1991(1253, 2408)	0.61
Mixed meal (pmol/L x min)	1811(1099,2018)	2330(1691,2730)	0.15
Fat-rich meal (pmol/L x min)	2532 ± 332	2483 ± 222	0.89

Paracetamol

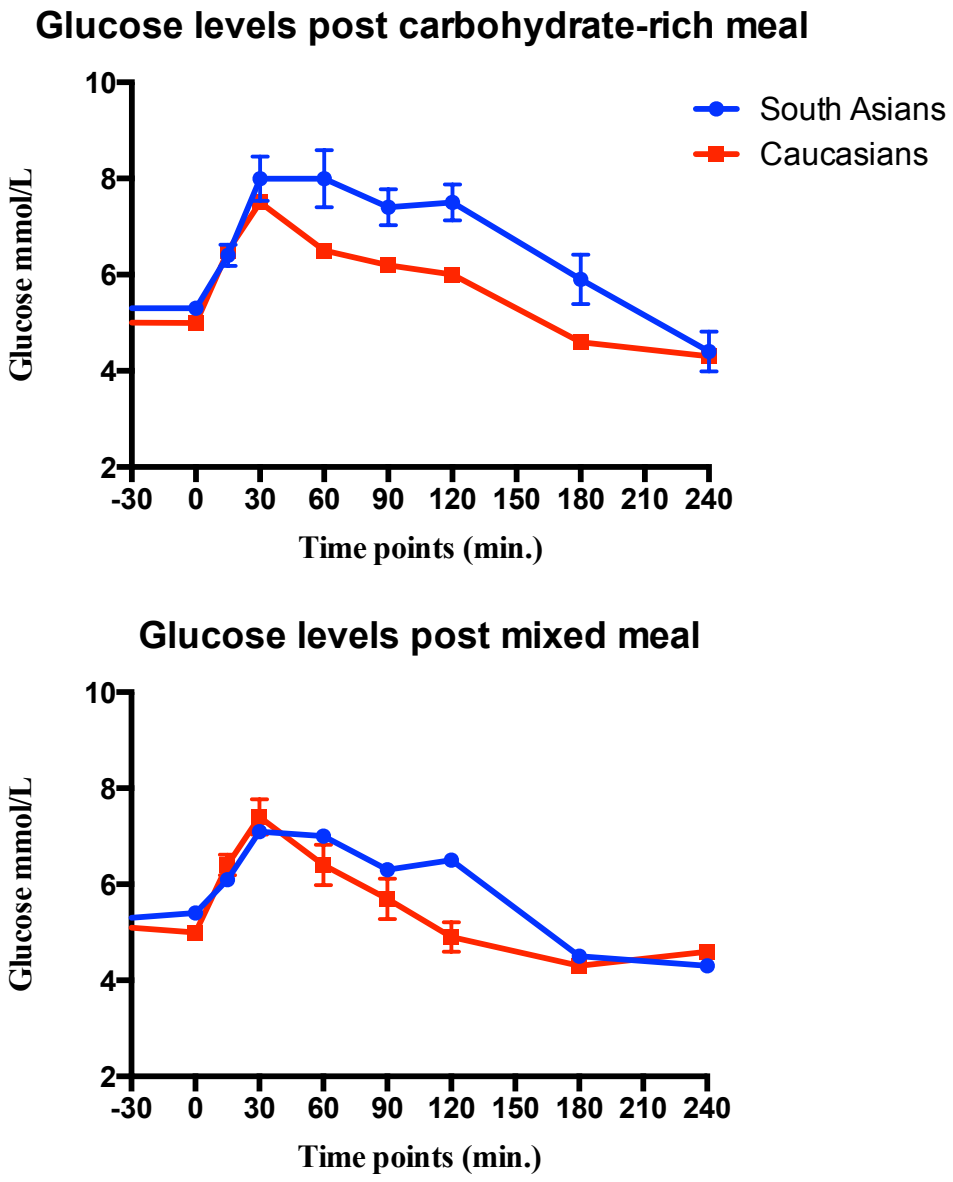
During our study, all participants ingested one gram of effervescent paracetamol with each of the three different meals.

The time to peak paracetamol levels (t_{max}) was higher in South Asians during carbohydrate-rich (SA at 90 min., CA at 60 min) and mixed meals (SA at 60 min., CA at 30 min.) while it was similar during fat-rich meals (at 60 min.).

Table 0.5 Summary table of key findings

	CHO-rich		Mixed		Fat rich	
	SA	CA	SA	CA	SA	CA
AUC_{240} GLP-1	2902±195	2908±256	3076±249	3097±254	2923±259	3053±235
AUC_{240} Insulin	20,162***	9636	12562	7044	5972	3373
AUC_{240} Glucose	1601±83.5	1361±44.1	1383±58	1260±35	1254±39	1192±28
ISSI-2	39	38	34±2	33±2	26±2	26±2
Matsuda index	3.2±0.5	5.7±0.5**	4.2±0.7	5.7±0.5	5.2±0.6	8.7±0.9**
Nadir glucagon levels (pmol/L)	5.1±1.2	5.7±0.5	5.2±1	6.7±0.8	6.6±1.2	7.9±0.7
Peak GLP-1 (pmol/L)	21.5±1.4	19.6±1.9	24±2	24.1±2.2	22.1±2	23.4±2
Peak glucose level (mmol/L)	8	7.5	7.1	7.4	6.8	6.8
Peak glucose time (minutes)	30	30	30	30	30	30
Peak insulin (mU/L)	168.4	89.7	114.4	92.6	79	61.2
Peak insulin time (minutes)	120	30	60	30	60	30

Figure 0.3 Plasma Glucose responses to carbohydrate -rich, mixed and fat-rich meals in South Asian and Caucasian subjects. Data points represent mean value ±standard error of mean.



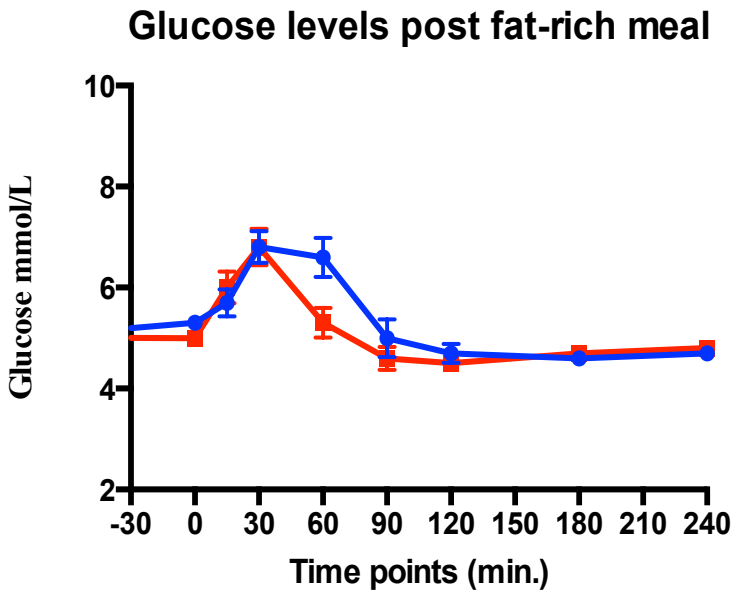

Glucose levels post fat-rich meal
Glucose mmol/L
10
8
6
4
2
-30
0
30
60
90
120
150
180
210
240
Time points (min.)

Figure 0.4 Integrated glucose responses expressed as total area under curve(AUC_{240}) values during carbohydrate-rich, mixed and fat-rich meal tests in South Asians and Caucasians

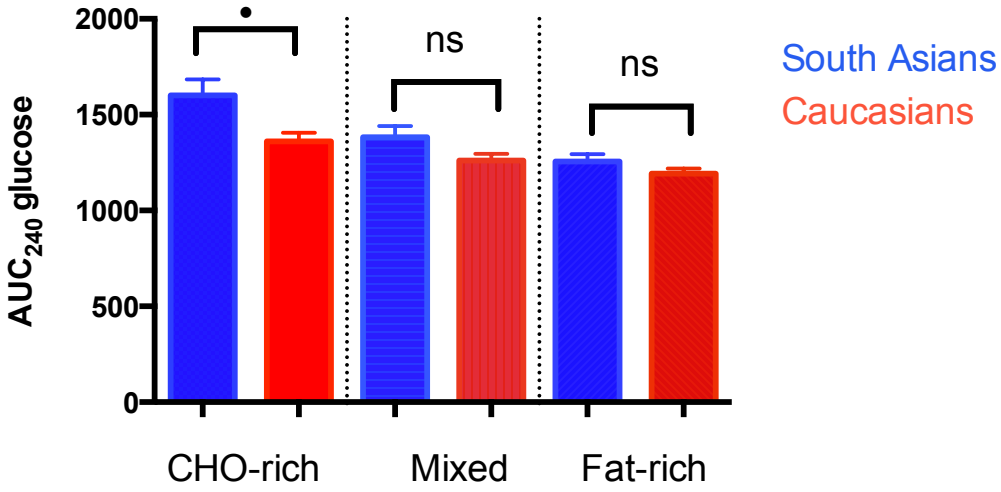


Figure 0.5 Insulin secretion rate (ISR) values in South Asians and Caucasians during carbohydrate-rich meals. Data points represent mean value ±standard error of mean.

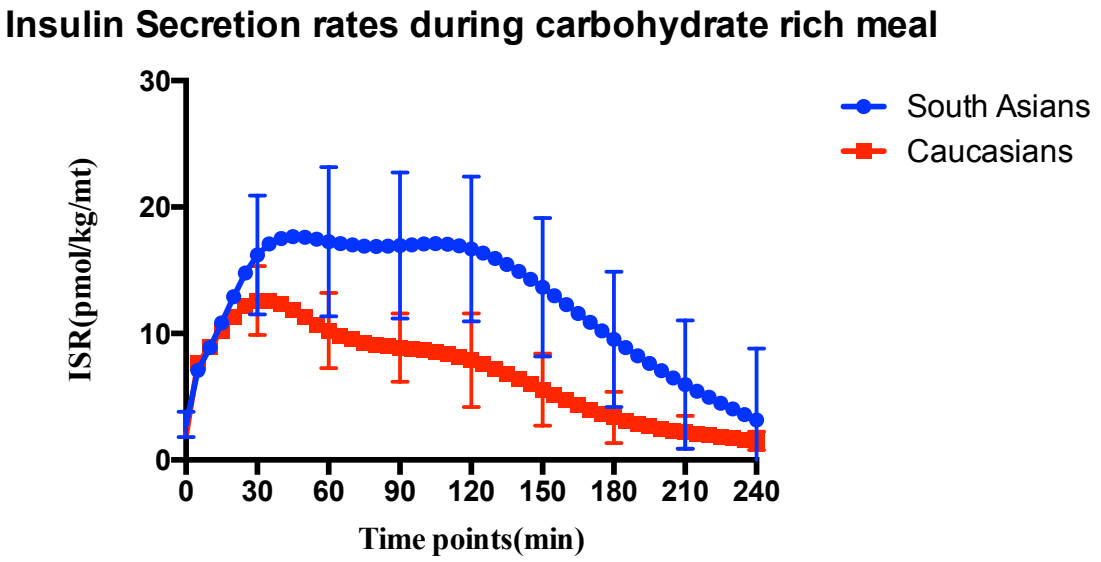


Figure 0.6 Insulin secretion rate (ISR) values in South Asians and Caucasians during mixed meals. Data points represent mean value ±standard error of mean.

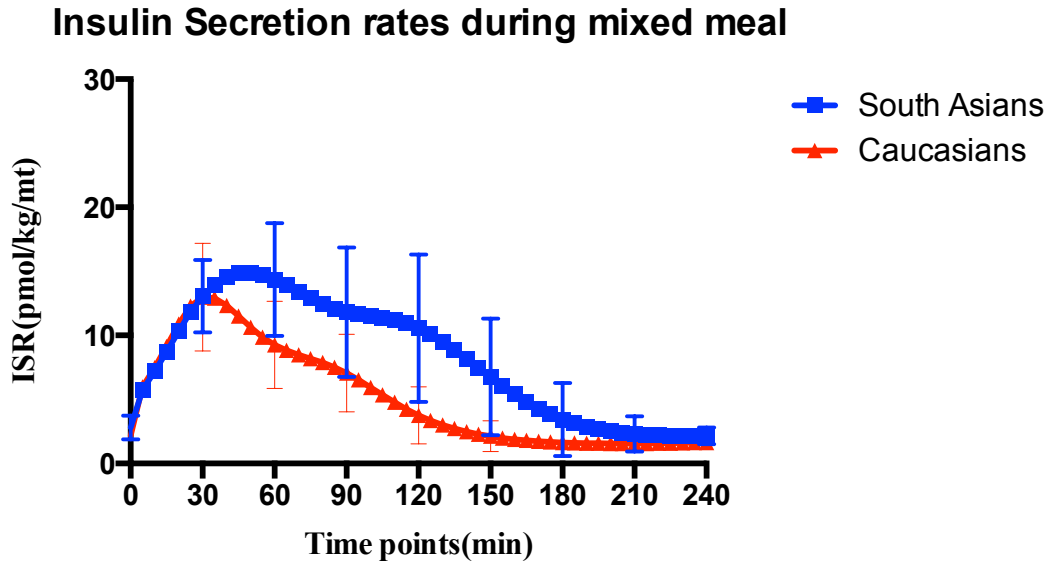


Figure 0.7 Insulin secretion rate (ISR) values in South Asians and Caucasians during fat-rich meals. Data points represent mean value ±standard error of mean.

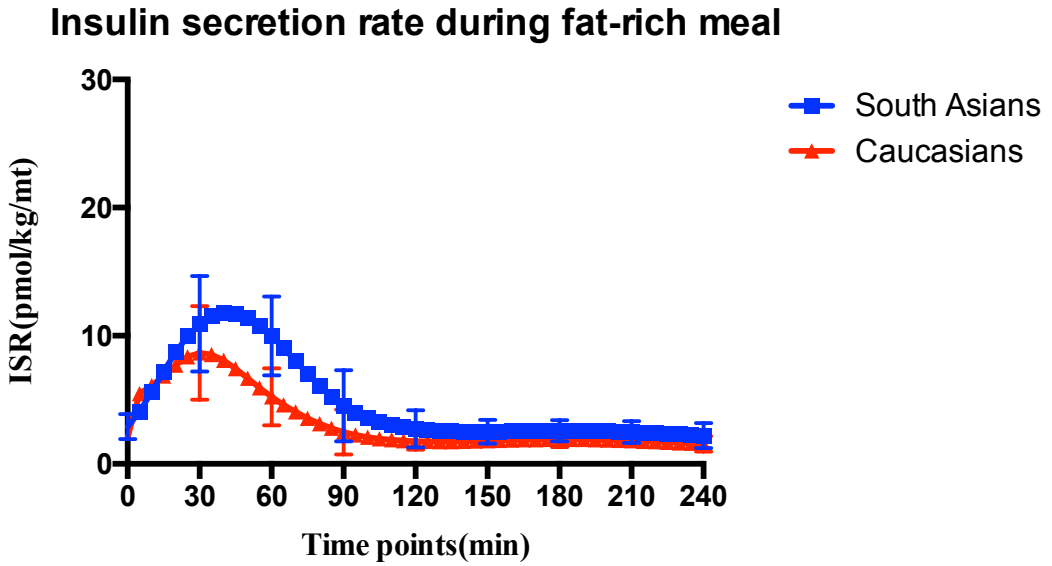
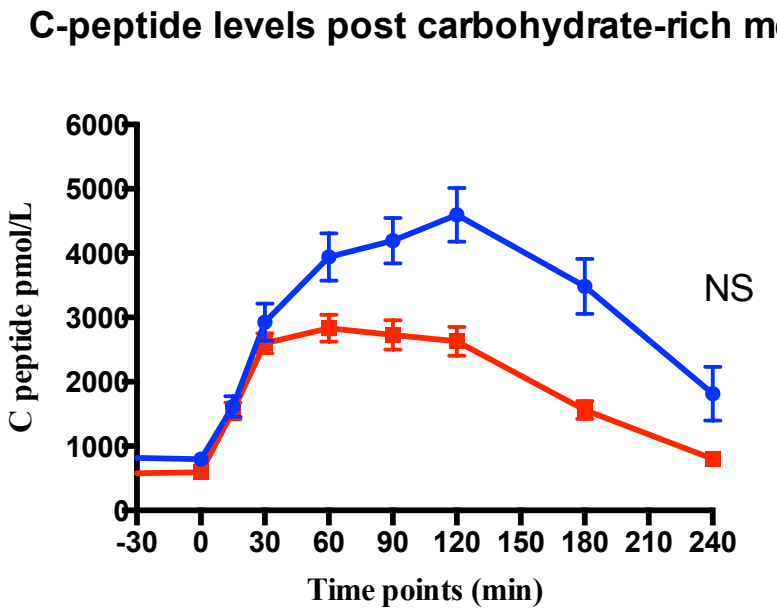
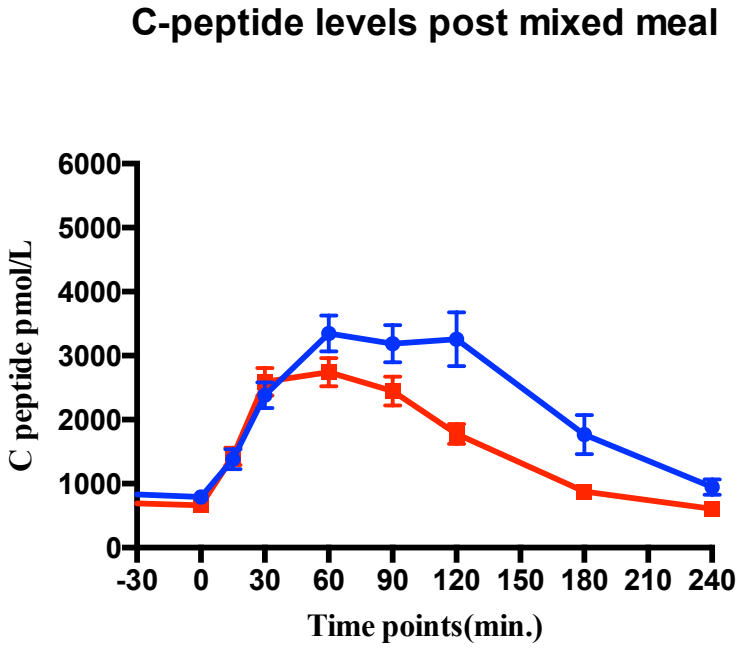


Figure o.8 C-peptide responses in South Asians and Caucasians during carbohydrate rich, mixed and fat-rich meals. Data points represent mean value ±standard error of mean.





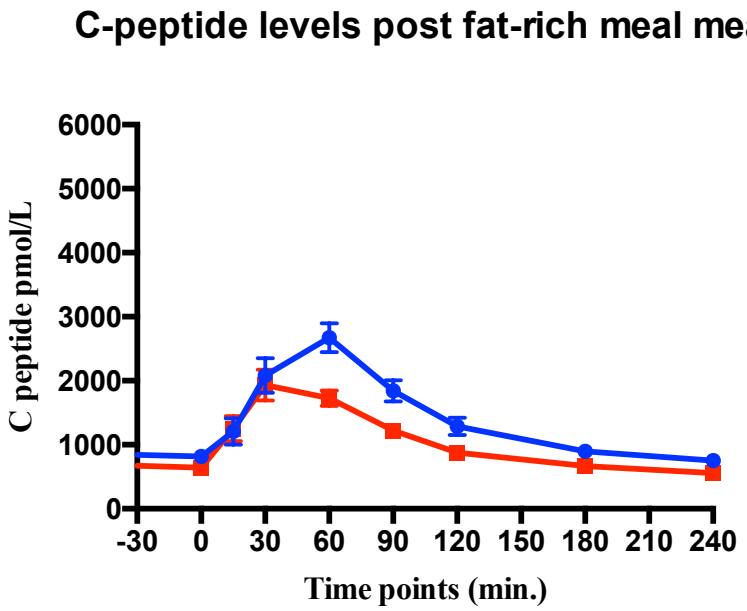
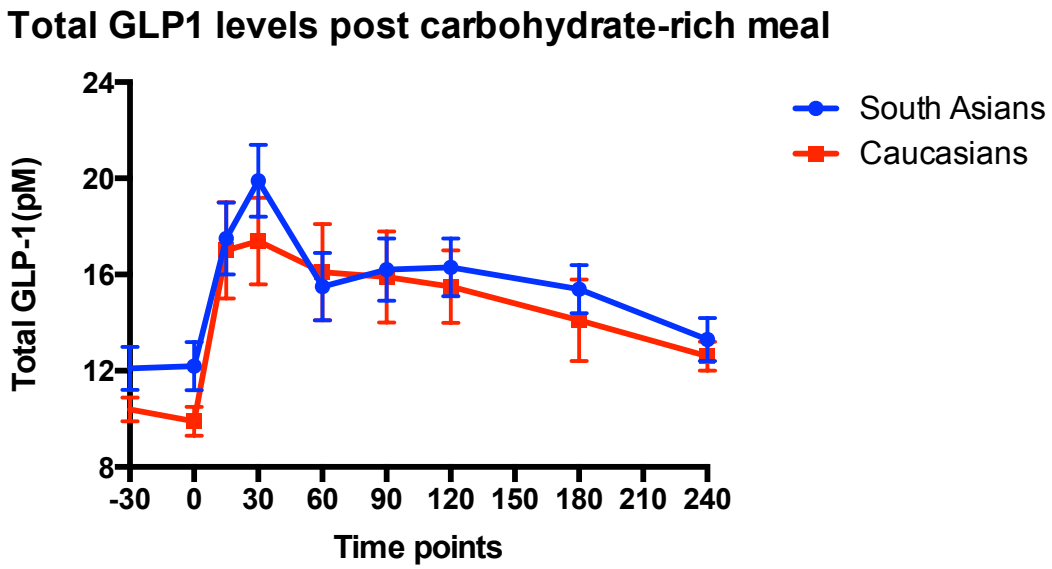
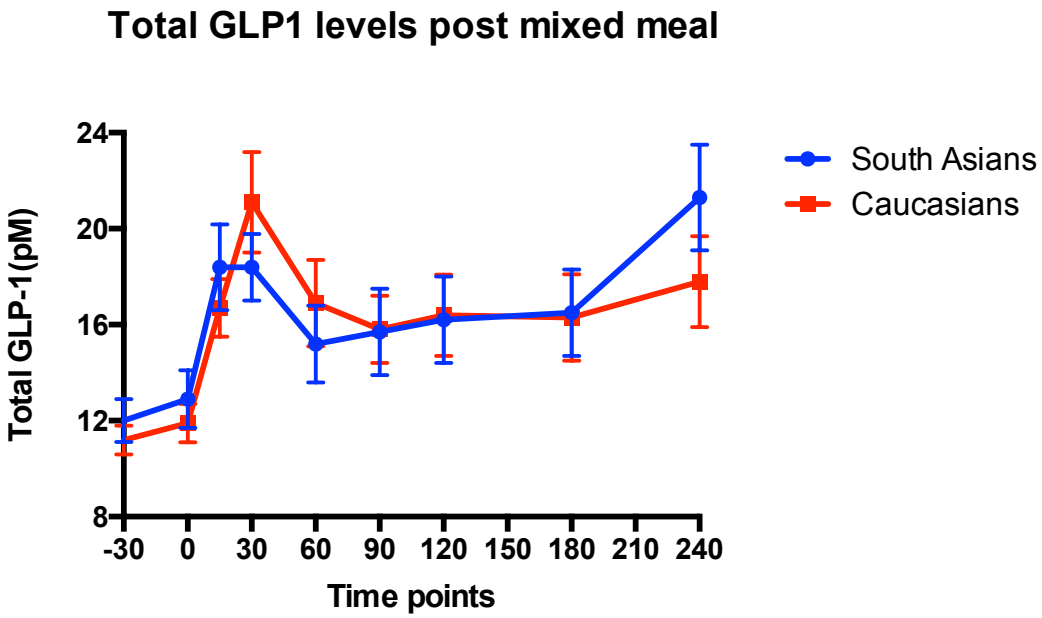

C-peptide levels post fat-rich meal meal
C peptide pmol/L
6000
5000
4000
3000
2000
1000
0
-30
0
30
60
90
120
150
180
210
240
Time points (min.)

Figure 0.9 Total GLP-1 responses to carbohydrate -rich, mixed and fat-rich meals in South Asian and Caucasian subjects. Data points represent value ±standard error of mean.





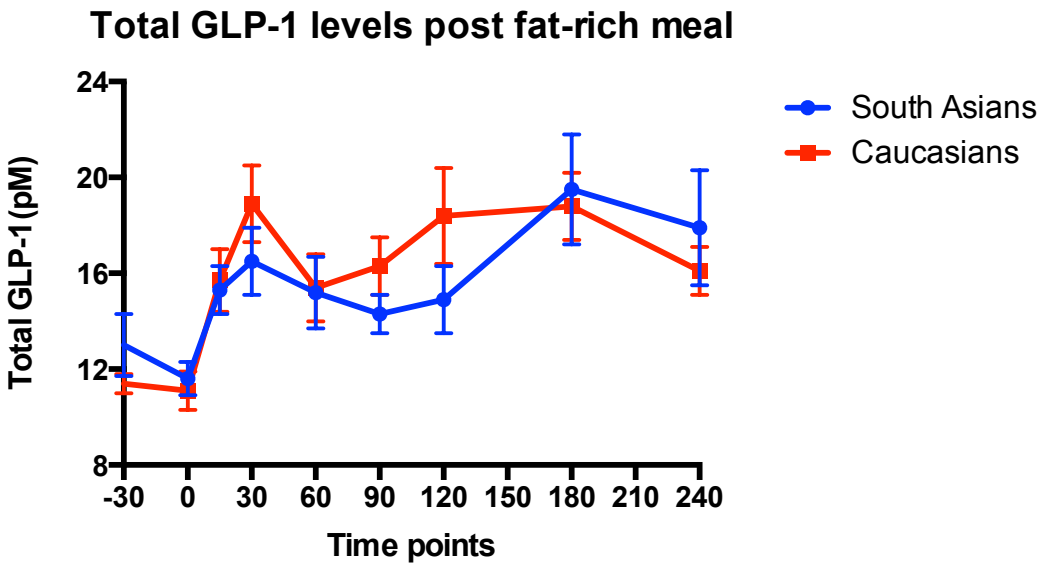
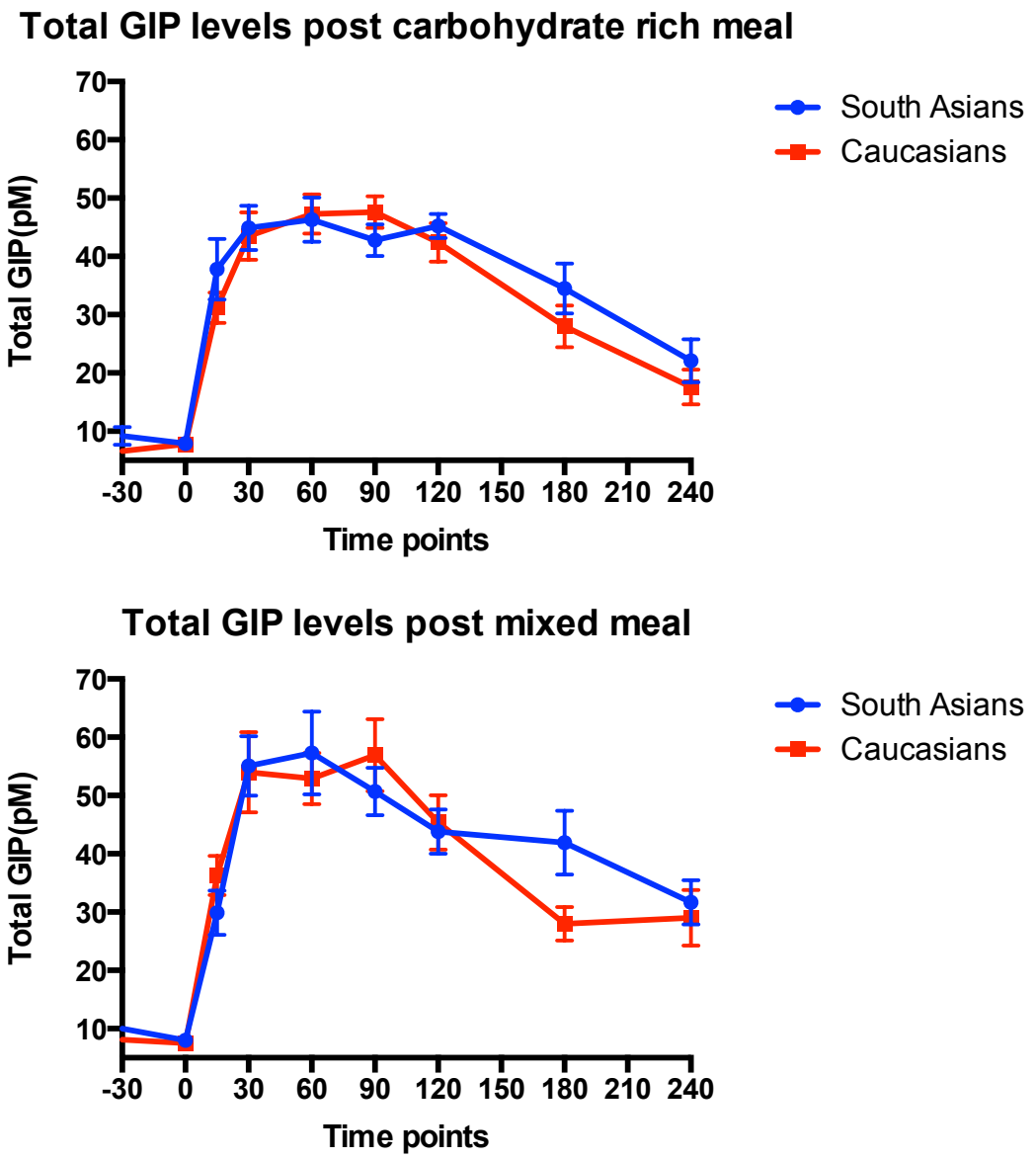

Total GLP-1 levels post fat-rich meal
Total GLP-1 (pM)
24
20
16
12
8
-30
0
30
60
90
120
150
180
210
240
Time points
South Asians
Caucasians

Figure 0.10 Total GIP responses to carbohydrate -rich, mixed and fat-rich meals in South



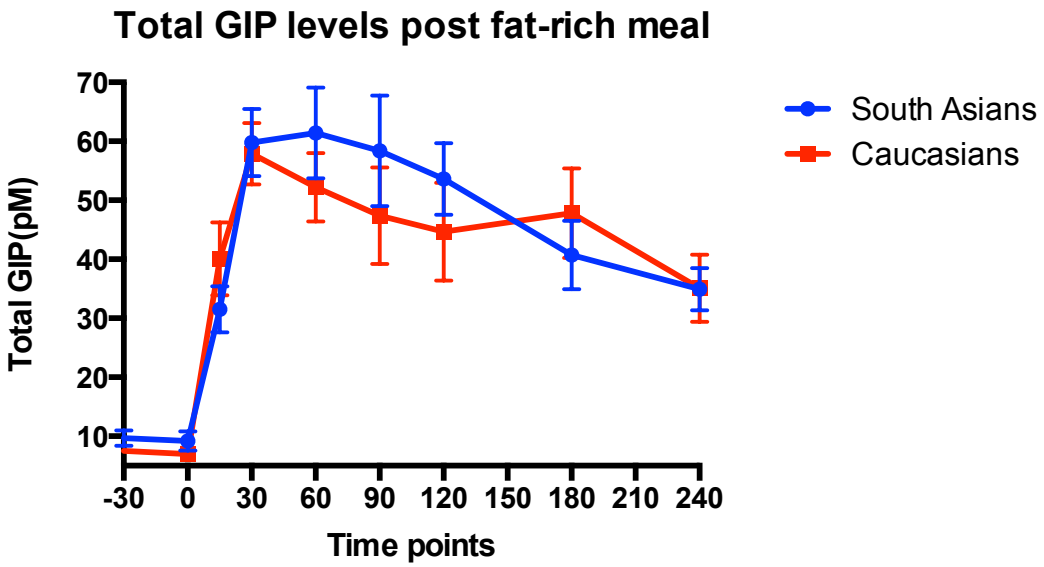
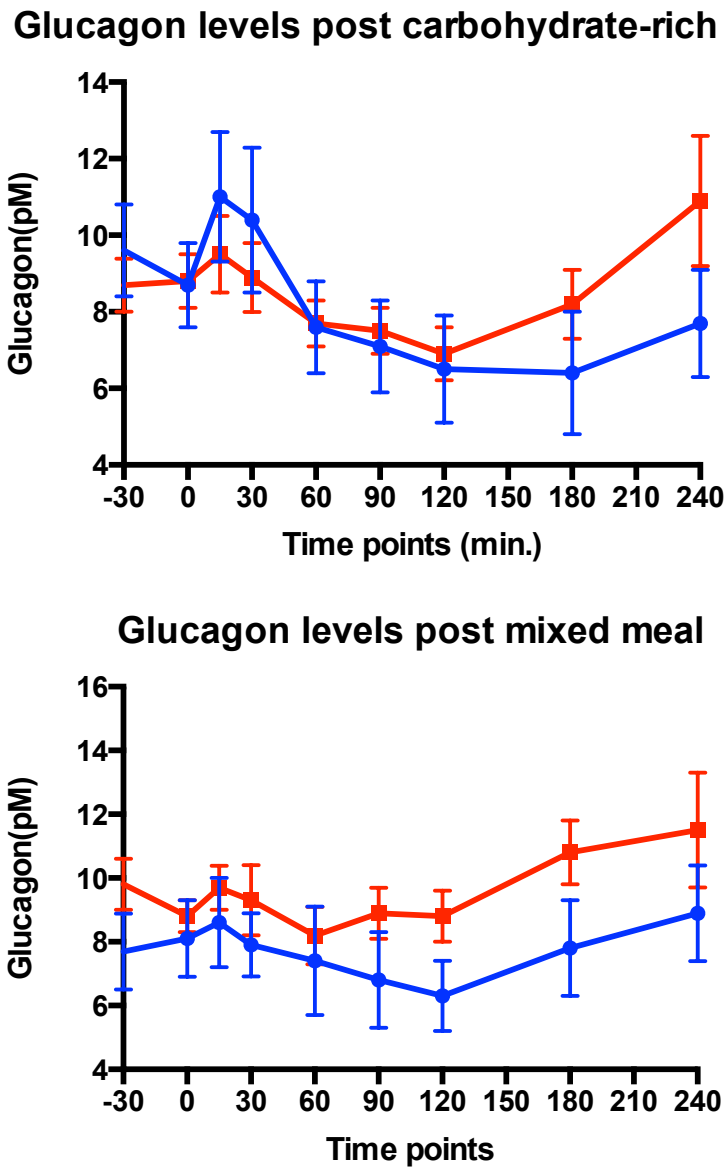
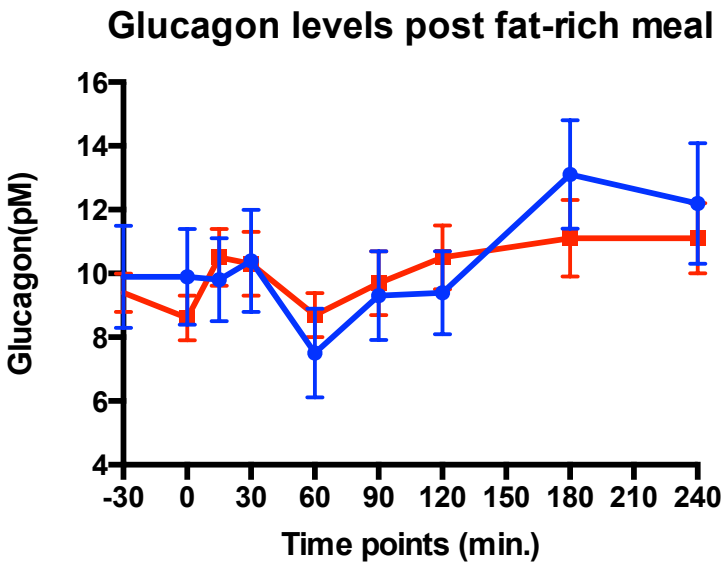

Total GIP levels post fat-rich meal
Total GIP(pM)
70
60
50
40
30
20
10
-30
0
30
60
90
120
150
180
210
240
Time points
South Asians
Caucasians

Figure 0.11 Glucagon responses to carbohydrate -rich, mixed and fat-rich meals in South Asian and Caucasian subjects. Data points represent value ±standard error of mean.




Glucagon levels post fat-rich meal
Glucagon(pM)
16
14
12
10
8
6
4
-30
0
30
60
90
120
150
180
210
240
Time points (min.)

1.37 Discussion

In our study, fasting glucose levels were similar between South Asians and Caucasians. South Asian subjects in our study were less insulin sensitive as demonstrated by the Matsuda index, a composite marker of (hepatic and peripheral) insulin sensitivity.

However South Asians demonstrated an overall higher incremental insulin response following different meal stimuli compared to Caucasians. In addition, the insulin secretion rates were also higher amongst South Asians. These differences were more pronounced during the second phase (t30-240 mins) in comparison to the first phase (t0-30 mins).

First phase insulin response is a result of hormone release from stored granules. The insulinogenic index, a marker of early insulin secretion (ratio of increment of insulin concentration to that of glucose) was also similar amongst both groups. However, this only takes into account the rise in insulin and glucose levels from baseline to 30 minutes (first phase) and hence is unsurprisingly similar between both groups. As the 0-30 min incremental glucose and insulin responses including insulin secretion rates were similar between South Asians and Caucasians in our study, the insulinogenic index was also as a result, similar between both groups. Hence a similar insulinogenic index suggests that South Asian subjects demonstrate sufficiently augmented insulin secretion compared to Caucasians despite the presence of reduced insulin sensitivity. Similar insulinogenic index in healthy subjects, despite presence of glucocorticoid induced insulin resistance has previously been reported as well (293).

Fasting total GLP-1 levels were higher in South Asians. Higher fasting GLP-1 levels in individuals with type 2 diabetes in comparison to those with normal glucose tolerance, has previously been reported ((169) (294).

Previous studies have shown the effects of meal size and composition on incretin hormone responses. One of the seminal studies was a randomised crossover study including subjects with type 2 diabetes and those with normal glucose tolerance (132).

The study was aimed at comparing effects of three different meals i.e. small carbohydrate rich, large carbohydrate rich and a fat rich meal.

Our findings suggest that NGT South Asians are less insulin sensitive compared to Caucasians with similar age and BMI, while demonstrating compensatory response to insulin resistance with augmented insulin secretion. The insulin responses (including both circulating insulin levels and the insulin secretion rates) were significantly higher in South Asians in comparison to Caucasians, despite comparable glucose levels during mixed and fat-rich meals. On the other hand, following a carbohydrate-rich meal, both glucose levels and insulin responses were significantly higher amongst South Asians. Altered glucose disposal following a carbohydrate-rich meal is likely due to loss of adaptive insulin response secondary to relative dysglycaemia. This is also congruent with previous studies reporting higher glycaemic response following carbohydrate rich meals in non-Caucasians (292) (289).

Despite higher basal GLP-1 levels, there was a lack of a similar post prandial response amongst South Asians. Therefore, unlike the insulin responses in the presence of insulin resistance, there was no evidence of an augmented total GLP-1 response post meal amongst South Asians.

The endogenous incretin hormone responses demonstrate discordance in relation to GIP and GLP-1 levels. While the total GLP-1 levels are fairly comparable in both groups, the total GIP levels were on the other hand higher amongst South Asians, during the late phase of insulin response (t30-240). This is similar to the exaggerated GIP response noted by Hansen et al following glucocorticoid induced insulin resistance (274).

Nonetheless the differences in postprandial incretin hormone levels are non-significant, in both our groups. Therefore, in the presence of demonstrable hyperinsulinaemia, this raises the possibility of a defect in 'adaptive incretin response' amongst South Asians. It may also raise a question of whether the compensatory hyperinsulinaemia in normal glucose tolerant South Asians occurs independent of the increase in the incretin hormone responses.

Adaptive incretin response following macronutrient ingestion, has previously been demonstrated both in fasting (295, 296) and postprandial states (296). The study by Numao et al, was in normal glucose tolerant Japanese subjects whereby short term low carbohydrate/high fat diet demonstrated suppressed insulin concentration during a 75-gram OGTT despite postprandial hyperglycaemia (296).

On the other hand, an impaired incretin effect has been observed following experimentally induced insulin resistance and glucose intolerance, without attenuation of GLP-1 or GIP levels (274). Investigators from the same group have also demonstrated impaired insulinotropic effects of GIP and GLP-1 after inducing glucose homeostasis dysregulation following a 12 day intervention involving sedentary lifestyle, high calorie meals and glucocorticoids (297).
The study by Bakker et al (196) showed impaired insulin sensitivity following ingestion of five days of high fat high calorie diet by young, lean, normal glucose tolerant South Asians in comparison to matched Caucasians. The results demonstrated a reduction in insulin-stimulated glucose disposal rate amongst South Asians but not in Caucasians.

As studies by Hansen et al (274, 297) and Bakker et al (196)were undertaken in Caucasians with no increased predisposition to develop type 2 diabetes, it is difficult to extrapolate these findings to South Asians. At the same time, insulin resistance induced by glucocorticoids is likely to have a different mechanism and consequent effect on glucose homeostasis and hence not directly comparable to endogenous de novo insulin resistance found in South Asians.

Variations in gastric emptying may be another factor underpinning the discrepancy in incretin hormone responses in our study. Rate of gastric emptying is also known to influence postprandial GLP-1 levels. Increased gastric emptying results in augmented postprandial GLP-1 response in normal glucose tolerant individuals.
There is increasing evidence supporting the influence of gastric emptying on incretin responses including the incretin effect (298). Marathe et al (298) have demonstrated the influence of the size of incretin effect is dependent on the small intestinal glucose load which in turn is determined by rate of gastric emptying.

Alsalim et al (299) have demonstrated increased GIP and GLP-1 responses to increasing meal sizes with the adaptation resulting in identical glucose excursions, in healthy lean subjects. The same groups have also studied (299) an integrative impact of macronutrients on postprandial glycaemia including incretin hormones. The group studies healthy subjects and those with type 2 diabetes (drug naïve) following ingestion of macronutrients alone or together. Mixed meal challenge diminished glucose excursions compared to glucose challenge alone. GLP-1 and GIP responses were increased after all challenges while GIP responses were markedly higher after the mixed meal rather than glucose alone.

There have been no previously reported studies making contemporaneous comparisons of postprandial incretin hormone responses between South Asians and Caucasians. Our study sheds further light on our evolving understanding of the incretin concept amongst South Asians. However, there were various limitations of our study. We had a small sample size which would potentially influence the significance of difference detected between groups. We also included participants from a broad age range as well as both genders. Both age and gender are known to be predictors of GLP-1 levels and hence this would have also potentially influenced our findings.

Chapter 5
Comparing Incretin Effect

Experiment one and two

Introduction

The incretin effect accounts for 50-70% of the postprandial insulin response. The two gastrointestinal hormones currently known to convey the incretin effect are GLP- 1 and GIP (120), constituting the endocrine part of the entero-insular axis as described by Unger et al (129).

Incretin effect is influenced by degree of dysglycaemia, obesity, NEFA and glucagon levels. In addition the incretin effect has been demonstrated to be a dynamic adaptive response which upregulates in the presence of increasing glucose load (173).
The incretin effect is reduced in patients with type 2 diabetes (172).
Evidence so far suggests that in type 2 diabetes, reduced incretin effect is due to a reduced GLP-1 response rather than reduced secretion.

1.38 Factors influencing the endogenous incretin effect:

Insulin resistance

1.38.1.1 Steroid induced insulin resistance

Effects of glucocorticoid induced insulin resistance is similar to the insulin resistance present in type 2 diabetes. Similar to endogenous insulin resistance in type 2 diabetes, glucocorticoids also result in reduced insulin-mediated peripheral glucose disposal (300, 301) , reduced oxidative and non-oxidative pathways of glucose disposal (300), reduced muscle glycogen synthase activity (302) and hepatic insulin resistance (301). Hence for these reasons, glucocorticoid induced insulin resistance, is a good model to study the potential interplay of various hormones in the context of type 2 diabetes. At the same time, while insulin resistance induced by glucocorticoids is similar to endogenous insulin resistance, its impact on insulin sensitivity and beta cell impairment is not exactly the same. The impact of chronic exposure to glucocorticoids also leads to increased site-specific adiposity. As known from studies in people with Cushing's syndrome, chronic glucocorticoid exposure leads to increased total and visceral adipose tissue mass but modest rise in subcutaneous depot (303). On the other hand, overall body fat distribution in South Asians is also different with propensity for fat deposition in lower rather than upper body(42, 76). Increased ectopic fat deposits in the dorso-cervical and under the chin area, have been reported to be a novel phenotype marker suggestive of increased risk of metabolic syndrome in South Asians (76).

The incretin effect is reduced in individuals with dexamethasone induced insulin resistance even with normal glucose tolerance (304). This is further diminished when they become glucose intolerant. The investigators studied healthy first-degree relatives of subjects with type 2 diabetes, using dexamethasone. They demonstrated that steroid induced insulin resistance had no impact on incretin hormone secretion (GLP-1 or GIP) during OGTT. Therefore, the reduced incretin effect in the context of

steroid induced insulin resistance has not been proven to be secondary to a reduced secretion of incretin hormones.

1.38.1.2 Obesity

Obese individuals with insulin resistance have been shown to have an impaired incretin effect as one of the earliest signs of metabolic perturbations (255).

Impaired insulinotropic effects of GLP-1 and GIP

Hansen et al (274) showed increased postprandial GIP but not GLP-1 response following steroid induced insulin resistance. In this study, Hansen el al induced insulin resistance in healthy subjects using dexamethasone, relative physical inactivity and high calorie diet in healthy subjects. A total of ten healthy Caucasian subjects with no family history of diabetes, were studied before and after intervention which included high calorie diet, relative physical inactivity and administration of prednisolone (37.5 mg/d) for 12 days. Pre and post intervention responses were compared following a liquid test meal.

Post intervention insulin resistance was confirmed according to the homeostatic model assessment, fasting plasma insulin, post prandial glucose excursions as well as insulin responses. However the increased insulin resistance had no impact on postprandial GLP-1 responses (AUC, 1.5 ± 0.7 *vs.* 2.0 ± 0.5 nM · 4 h; $P = 0.56$), but did result in exaggerated postprandial GIP (6.2 ± 1.0 *vs.* 10.0 ± 1.3 nM · 4 h; $P = 0.003$) and glucagon responses (1.6 ± 1.5 *vs.* 2.4 ± 3.2; $P = 0.007$).

Eriksen et al (305) studied first-degree relatives of subjects with type 2 diabetes. They induced glucose intolerance with the use of dexamethasone, to improve understanding of impaired incretin effect in the context of insulin resistance. The main findings of the study were that when measured as first phase responses (glucose clamp at 7 mmol/mol) the insulinotropic responses of the two incretin hormones GLP-1 and GIP, did not change. However, post dexamethasone, the second phase responses at the same glucose clamp were significantly impaired. Similar findngs when comparing beta cell secretory capacity to arginine (maximal insulin responses at 15 mmol/mol).

The same group has later examined the insulinotropic effects of GLP-1 and GIP in the context of dexamethasone induced insulin resistance (177).

Eriksen et al (305) studied insulinotropic responses of GLP-1 and GIP in first degree relatives of those with type 2 diabetes during glucose clamp experiments. The investigators studied the responses at two different glucose levels, 7 mmol/l and 15 mmol/l to capture responses at both physiological and pathologically elevated glucose levels. This study also evaluated whether a reduced incretin effect was a result of maximal secretory response using arginine, a non-glucose, non-incretin beta cell stimulus. It was noted that dexamethasone did not impair beta cell function as the secretory capacity following arginine, was not altered.

1.39 Incretin effect in South Asians

We know that in comparison to Caucasians, South Asians have evidence of insulin resistance from very early years of life, even at lower levels of adiposity. However, what is not known yet is whether the incretin effect per say, is different in South Asians, in comparison to Caucasians. Both mechanistic and drug efficacy studies involving comparison of incretin effect amongst South Asians, have been discussed at length in the introduction section. However, a majority of these studies include subjects from Far East Asia rather than those of South Asian descent. Far East Asians, in contrast to South Asians, have a predominant insulin secretory defect rather than insulin resistance, driving their increased risk of type 2 diabetes (237).
Similarly there have been studies looking at the effects of steroid induced insulin resistance on the incretin effect (306) but findings from these studies again cannot be extrapolated in entirety given that metabolic changes due to short term steroid induced insulin resistance are not the same as those resulting due to endogenous de novo insulin resistance in South Asians.

There is also preliminary evidence to suggest that in comparison to Caucasians, the efficacy of incretin-based drugs vary amongst South Asians.

To the best of our knowledge, there is no reported evidence to date (at the time of undertaking the research studies), directly comparing the incretin effect between normal glucose tolerant South Asians and Caucasians.

1.40 Subjects and methods

Subjects

Ten South Asian subjects (age: 33±4 yrs; BMI: 24.5±1 kg/m^2;
fasting plasma glucose: 4.8±0.2 mmol/L) and twelve age and BMI Caucasian subjects (age: 31±3 yrs; BMI: 24.6±1 kg/m^2; fasting plasma glucose: 4.6±0.1 mmol/L) with normal glucose tolerance, were recruited. Normal glucose tolerance was confirmed following a 75-gram oral glucose tolerance test (OGTT), as per WHO criteria, which was performed prior to recruitment. None of the subjects had anaemia (Haemoglobin <12.0 g/dL), impaired renal (eGFR<60 mls/min/1.73m^2) or liver (ALT, AST, ALP or GGT greater than 2 times upper limit of normal) function tests or proteinuria. All subjects gave written consent for participation after receiving oral and written study information.

Methods

All subjects attended following a ten hour overnight fast (water was permitted during fasting state). All participants abstained from exercise for 48 hours prior to each visit. All subjects were also advised to consume 'usual meal' at least for 48 hours prior to the experiment

All studies were performed at the Clinical Research Facility at the Royal Liverpool & Broadgreen University Hospitals NHS Trust. The study included two visits (experiment one and two) which were performed in a fixed order. The 50-gram oral glucose tolerance test (OGTT) (experiment 1) was followed by the isoglycaemic clamp study. Both experiments were conducted at least 48 hours apart.

In this chapter we report findings of part one of our study, aimed at comparing quantitative differences in the incretin effect between age and BMI-matched South Asians and Caucasians.

The incretin effect, in our study, was measured using both conventional and non-conventional methods. The various parameters used for comparing the incretin effect were C- peptide, insulin secretion rate using deconvolution method and gut mediated glucose disposal (GIGD).

Study protocol

All subjects were studied on two different occasions at least 48 hours apart.
All subjects abstained from exercise for 48 hours prior to each experiment and followed their 'usual' diet during this period. Also, subjects omitted any regular medications including over the counter drugs for 24 hours prior to each experiment.

For each occasion, subjects were studied in the morning following an overnight 10-hour fast. On arrival, all subjects were weighed (TANITA body composition analyser BC-420MA) followed by an ambulatory blood pressure measurement.

A cannula was inserted in the cubital vein for collection of arterialized blood samples. The arm was kept warm using a heating pad.

1.40.1.1 OGTT:

On the first visit, participants underwent a 4-hr OGTT (50 g) with blood glucose monitoring at 5-minute intervals. During both visits, arterialized blood was sampled at -15, 0 and 15, 30, 45, 60, 90, 120, 180 and 240 minutes.
The cannulated arm was kept warm using a heating pad in between samples.
Blood glucose was measured using an YSI STAT analyser.

Figure 0.1 Experiment work sheet for visit one (OGTT)

ISSA-4149 Study part 1

OGTT- Visit 1

Date: Fasting? Medications omitted?

BP: HR:

Weight: BMI:

% Body fat: Fat free mass:

Any change since recruitment/last visit?

Time	Timer	Real Time	Samples	BG(1)	BG(2)	BG (mean)	Notes
-15	0		G, Add				
-10	5		G, Add				
0	15		G, Add				
0	**15**		**Lucozade 273 mls.(50 gm glucose)**				
5	20		G				
10	25		G				
15	30		G, Add				
20	35		G				
25	40		G				
30	45		G, Add				
35	50		G				
40	55		G				
45	60		G, Add				
50	65(1.05)		G				
55	70(1.10)		G				
60	75(1.15)		G, Add				
75	90(1.30)		G, Add				
90	105(1.45)		G, Add				
120	135(2.15)		G, Add				
180	195(3.15)		G, Add				
240	255(4.15)		G, Add				

G: YSI (0.5 ml)-glucose

ADD:

Pink EDTA (1 tubes with DA chilled) - 7 mls. (GIP+ GLP-1+ EXTRA(x2)),

Pink EDTA (1 tube with A chilled) - 2 ml (Glucagon + EXTRA); Orange heparin- 1ml (EXTRA)

White Serum (1 tube) - 5 mls (Insulin/C-pep, triglycerides+ FFA + EXTRA)

D= DPP4i, A=Aprotonin (500 KIU/ml blood), Centrifuge@ 4 deg, 4000 rpmx10 mins

Each plus (+) denotes a separate aliquot

1.40.1.2 IGII: Isoglycaemic clamp

The plasma glucose profile of the individual subject, following a 50-gram OGTT, was reproduced using 20% dextrose infusion. Glucose infusion rates were adjusted based on the 5-minute glucose readings to mimic the glucose levels achieved following the 50 g oral glucose load.

Figure 0.2 Experiment work sheet for visit 2(IGII)

ISSA-4149 Study part 1

IGII- Visit 3

Date: Fasting? Medications omitted?

BP: HR:

Weight: BMI:

% Body fat: Fat free mass:

Any change since recruitment/last visit?

Time points	Timer	Real Time	Sample	BG1	BG2	BG mean	Glucose @ ml/hr	Total glucose	Time
-15	0		G, Add						
-10	5		G, Add						
0	15		G, Add						
5	**20**		G	**Start 20% Dextrose infusion**					
10	25		G						
15	30		G, Add					*	
20	35		G						
25	40		G						
30	45		G, Add					*	
35	50		G						
40	55		G						
45	60		G, Add					*	
50	65(1.05)		G						
55	70(1.10)		G						
60	75(1.15)		G, Add					*	
75	90(1.30)		G, Add					*	
90	105(1.45)		G, Add					*	
120	135(2.15)		G, Add					*	
180	195(3.15)		G, Add					*	
240	255(4.15)		G, Add					*	

G: YSI (0.5 ml)-glucose

ADD:

Pink EDTA (1 tubes with DA chilled) - 7 mls. (GIP+ GLP-1+ EXTRA(x2)),

Pink EDTA (1 tube with A chilled) - 2 ml (Glucagon + EXTRA); Orange heparin- 1ml (EXTRA)

White Serum (1 tube) - 5 mls (Insulin/Cpep, triglycerides+ FFA + EXTRA)

D= DPP4i, A=Aprotonin (500 KIU/ml blood), Centrifuge@ 4 deg, 4000 rpmx10 min

Each plus (+) denotes a separate aliquot

Laboratory analyses

Glucose levels were measured using the glucose oxidase method with a glucose analyzer (Yellow Springs Instrument Model 2300 STAT plus analyzer; YSI Inc., Yellow Springs, OH). The analyser was received on loan from Professor Dan Cuthbertson, Diabetes Centre, Aintree University Hospital, UK.

Both insulin and c-peptide levels were measured using an automated electrochemiluminescence immunoassay (ECLIA) analyser based on the sandwich principle (Roche Cobas E601; Roche Products Limited, Hertfordshire, UK). The lower detection limit for insulin is 0.2 milliunits/ml and 0.003nmol/L for C-peptide. Inter assay coefficient of variation for insulin and C-peptide are 1.76% and 2.3%. The cross-reactivity with intact and split proinsulin in the C-peptide assay was insignificant.

Triglycerides were measured using an automated COBAS analyser based on the enzymatic colorimetric test.

Samples for total GLP-1, GIP and glucagon were analysed at Professor Holst's laboratory at Panum Institute, Copenhagen Denmark. Plasma samples were assayed for total GLP-1 immunoreactivity using an antiserum that reacts equally with intact GLP-1 and the primary (N-terminally truncated) metabolite (144). Total GIP was measured using the C-terminally directed antiserum, which reacts fully with intact GIP and the N-terminally truncated metabolite, as previously described. The glucagon assay was directed against the C-terminal of the glucagon molecule and, therefore, measured glucagon of pancreatic origin (246) only.

Calculation and statistics

All results are expressed as means ± standard error of the mean (SEM) unless otherwise stated. Area under curve (AUC) was calculated using the trapezoidal rule. Incremental AUCs were obtained by deducting the area below baseline from total AUC. Insulin secretion rate (ISR) values were calculated by deconvolution of measured C-peptide concentrations and application of population-based parameters for C-peptide kinetics as described previously. ISR is expressed as picomoles insulin secreted per minute per kilogram body weight.

Normally distributed data were compared using paired two-tailed t tests. In case of data not following a normal distribution, significance of differences was tested using a Wilcoxon test for paired comparisons and Mann Whitney U test for unpaired comparisons. A p value of <0.05 were considered statistically significant.

Gastrointestinally mediated glucose disposal (GIGD)

Gastrointestinally mediated glucose disposal (GIGD) is calculated on the basis of the amount of intravenous glucose required to replicate the plasma glucose curve produced during a fixed oral glucose load ((GIGD (%) = 100%× (glucose oral - glucose iv)/glucose oral). It describes not only the impact of the incretin effect but includes all factors influencing glucose disposal differently based on route of administration (including neural reflexes, activation of afferent nerves in the intestinal mucosa, differences in glucagon secretion, hepatic glucose production and first-pass hepatic uptake of glucose, differences in portal and venous blood glucose concentrations and unknown factors).

Glucose disposal (during oral glucose tolerance test) following oral route of glucose administration can be evaluated by measuring GIGD, which in healthy subjects amounts to ~60%.

GIGD is one of the ways of evaluating the impact of the incretin effect on glucose disposal - in healthy subjects most of the GIGD is accounted for by the actions of incretin hormones (128).

For the purpose of our study, GIGD was calculated using the formula 100x ($glucose_{OGTT}$-$glucose_{IGII}$)/$glucose_{OGTT}$ with glucose $_{OGTT}$ being 50 gm. for all subjects in our study.

1.41 Results

Anthropometry

Both groups were comparable for age, gender and BMI. In addition, the waist hip ratios (WHR), visceral adiposity and body fat percentage (using BIA) were also non significantly different between both groups, at baseline (table 5.1).

Table 0.1 Baseline demographics and anthropometric data for both South Asians and Caucasians

	South Asians	Caucasians	p
N (female/male)	10 (4/6)	12 (7/5)	-
Age (years)	34 (22-56)	31 (21-55)	NS
BMI (kg/m^2)	24.5 (19.4-31.6)	24.6 (19.6-35)	NS
Fasting plasma glucose (mmo/L)	4.8 (4-5.8)	4.6 (4-5.1)	NS
75g-OGTT post load glucose (mmol/L)	4.8 (3-6.4)	3.8 (2.2-5.8)	NS
Waist-hip ratio (WHR)	0.85 (0.68-1.0)	0.83 (0.67-0.98)	NS
Visceral adiposity	5.4 ± 1.2	4.8 ±1.4	NS
Body fat (%)	24.2±3	24.9±2	NS

Glucose

1.41.1.1 Fasting

There were no significant differences observed in fasting glucose levels during experiment 1(OGTT) and experiment 2 (IGII) within both groups.
Mean fasting glucose level in South Asians was significantly higher compared to Caucasians (SA 4.7± 0.1 vs. CA 4.3±0.05 mmol/L; p=0.002).

1.41.1.2 Stimulated

Oral glucose curves were replicated during the adjustable intravenous glucose infusions (with no significant differences between the two glucose curves in both groups).

Within groups (OGTT vs. IGII)

South Asians
Peak glucose level achieved during OGTT was 10.4 mmol/L at 40 minutes. During IGII, the peak glucose achieved was 12.6 mmol/L at 45 minutes (figure 5.3).

Caucasians
Peak glucose level achieved during OGTT was 9.69 mmol/L at 45 minutes while during IGII, it was 10.1 mmol/L at 45 minutes (figure 5.4).

Figure 0.3 Plasma glucose responses in South Asians during OGTT (filled symbols) and IGII (closed symbols).

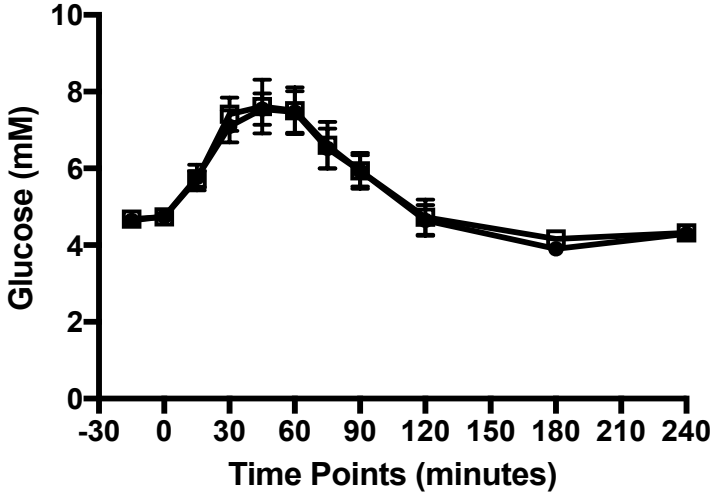
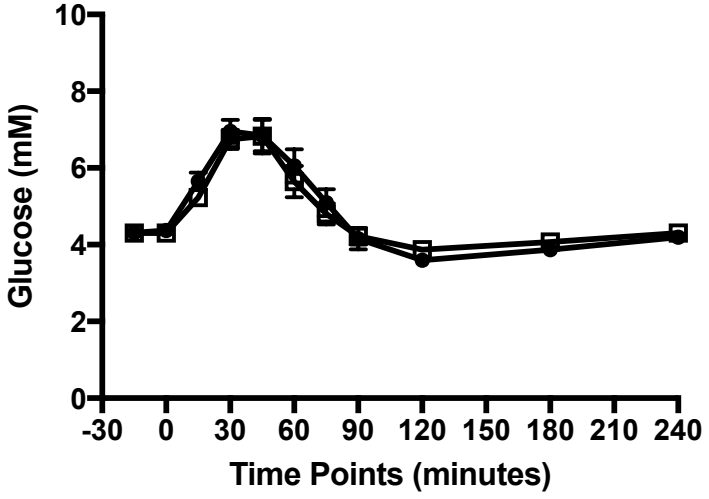


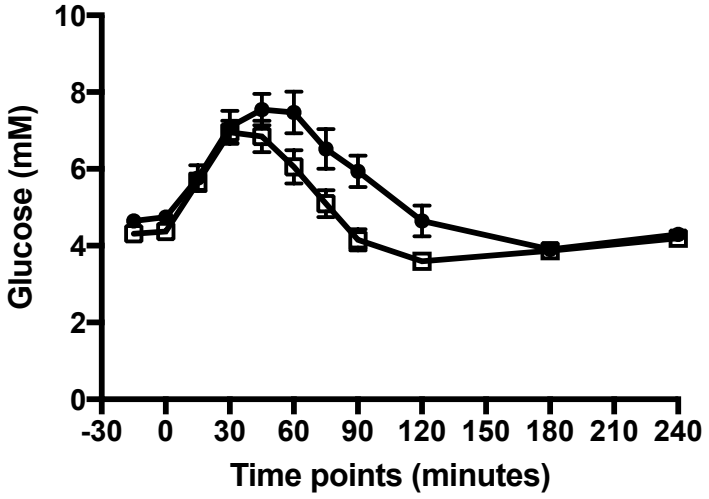
Figure 0.4 Plasma glucose levels in Caucasians during OGTT (filled symbols) and IGII (closed symbols).



Between groups (South Asians vs. Caucasians)

In South Asian subjects, glucose levels remained raised post loading during OGTT for much longer, in comparison to Caucasians (figure).

Figure 0.5 Plasma glucose levels in South Asians (filled symbols) and Caucasians (open symbols) during OGTT.



1.41.1.3 Glucose consumption

Overall glucose consumption during IGII was non-significantly higher in South Asians in comparison to Caucasians.

Figure 0.6 Glucose consumption during isoglycaemic intravenous glucose infusion(IGII), at individual time points in South Asian (top panel) and Caucasian subjects (bottom panel)

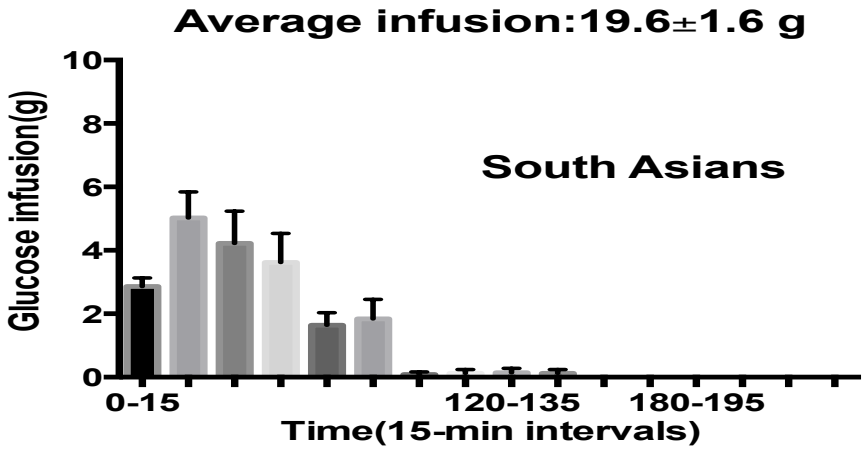
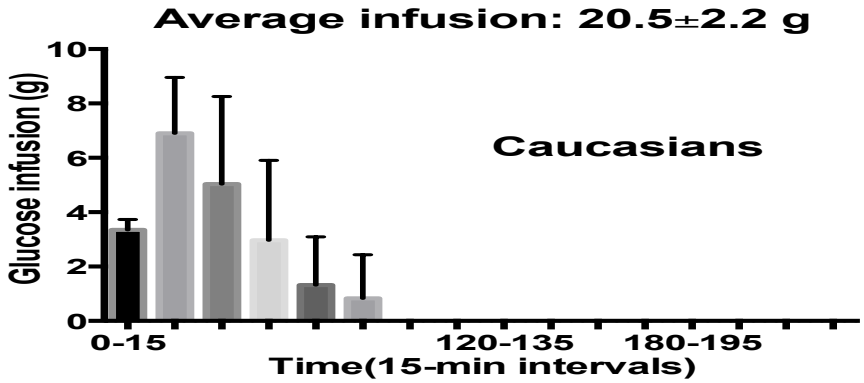


Figure 0.7



Insulin

1.41.1.4 Fasting

Mean fasting insulin levels in South Asians were significantly higher in comparison to Caucasians (12.0±1.6 vs. 8.0±0.8 mU/l, p=0.02).

1.41.1.5 Stimulated (OGTT vs. IGII)

Within groups

Integrated insulin responses (total AUC) during day one (OGTT) was significantly higher in comparison to day two (IGII) in both groups (SA $tAUC_{OGTT}$ 9367 vs. SA t AUC_{IGII} p=; CA t AUC_{OGTT} 5287 vs. CA t AUC_{IGII}, p=0.0009).

Figure 0.8 Plasma insulin responses in South Asian subjects during 50-gm OGTT (filled symbols) and IGII (open symbols).

Data points are mean±SEM

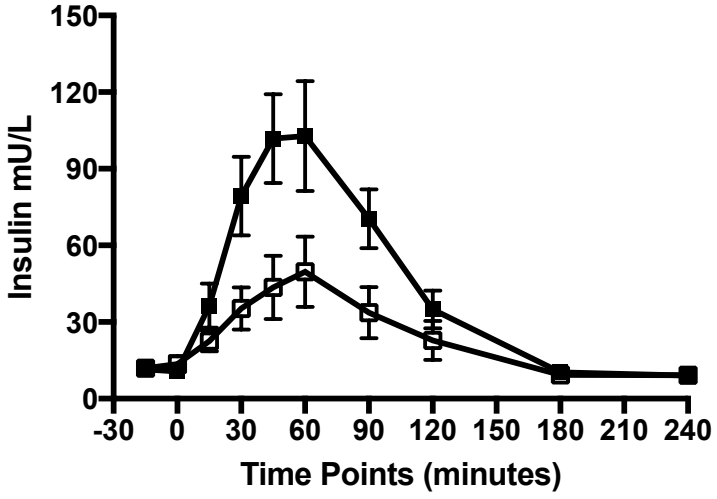
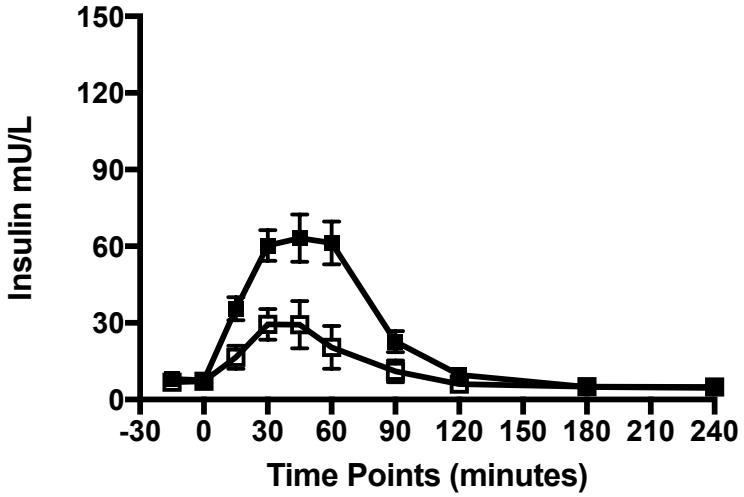


Figure 0.9 Plasma insulin responses in Caucasian subjects during 50-gm OGTT (filled symbols) and IGII (open symbols).

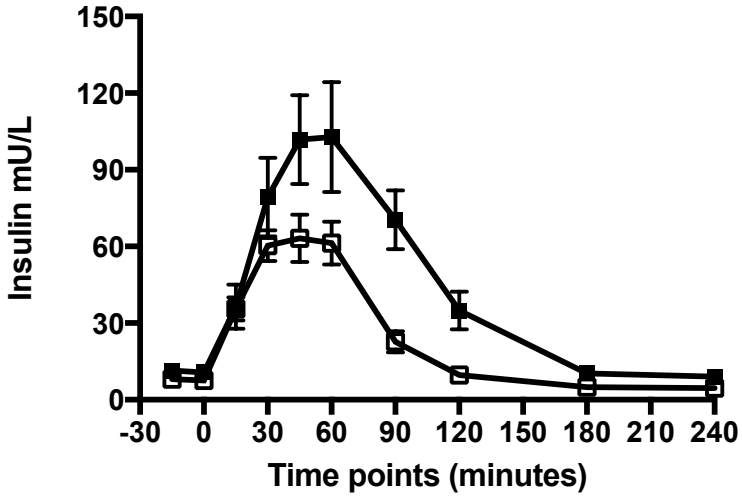
Data points are mean ± SEM.



Between groups (South Asians vs. Caucasians)

Integrated insulin responses (tAUC) during OGTT and IIGI, respectively, were significantly higher in South Asians compared to Caucasians (OGTT SA vs. CA: 9367 vs. 5287 mU/l x min, p=0.005; IGII: 5610±1347 vs. 2703±321 mU/l x min, p=0.03). Incremental insulin responses (iAUC) during OGTT were also significantly higher in South Asians compared to Caucasians (figure 5.10).

Figure 0.10 Plasma insulin responses in South Asians (filled symbols) and Caucasian (open symbols) during 50-gm OGTT.



C-peptide

1.41.1.6 Fasting

Mean fasting C-peptide levels in South Asians (including both values from OGTT and IGII) were significantly higher compared to Caucasians (Basal SA $_{CPEP}$ 489.6±63.66 vs. Basal CA $_{CPEP}$ 641.7±38.22 pmol/L, p=0.03).

1.41.1.7 Stimulated

Within groups (OGTT vs. IGII)

Integrated C-peptide responses (total AUC) during experiment one (OGTT) were significantly higher in comparison to experiment two (IGII) amongst both groups.

Figure 0.11 Plasma c-peptide responses in South Asians subjects during 50-gm OGTT (filled symbols) and IGII (open symbols).

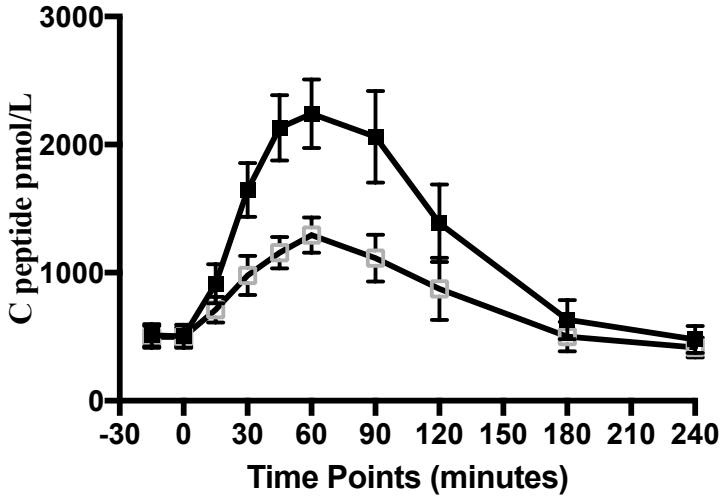
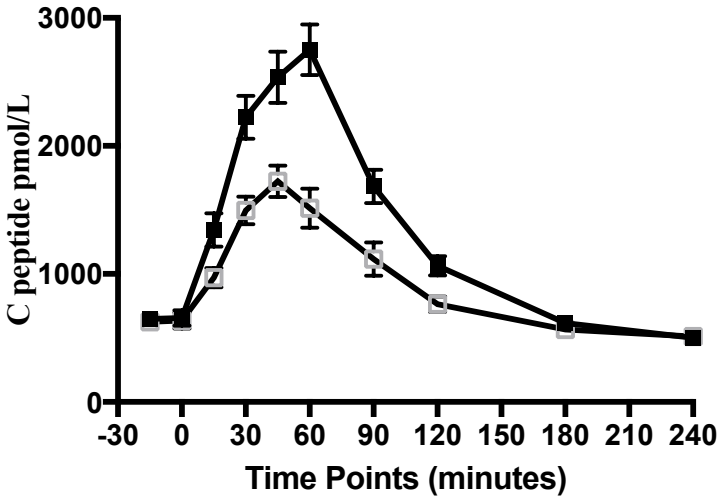


Figure 0.12 Plasma c-peptide responses in Caucasian subjects during 50-gm OGTT (filled symbols) and IGII (open symbols).

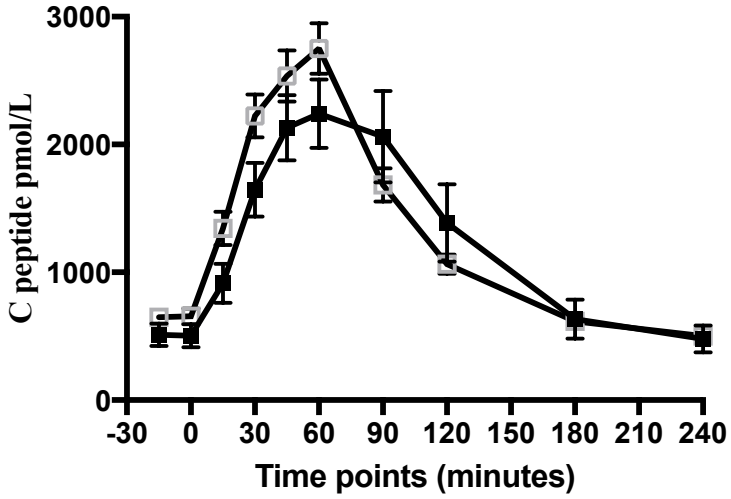


Between Groups

OGTT

There were no significant differences in the integrated C-peptide responses (tAUC) between South Asians and Caucasians (SA $tAUC_{OGTT}$: 352845±53954 vs. CA $tAUC_{OGTT}$: 350099±20144 pmol/L; p=0.95) (figure 5.13).

Figure 0.13 Plasma c-peptide responses in South Asians (filled symbols) and Caucasian (open symbols) during 50-gm OGTT.



Peak C-peptide level achieved in South Asians during OGTT was 3720 pmol/L at 90 minutes.

Glucagon- like Peptide-1

1.41.1.8 Fasting levels

Mean fasting total GLP-1 levels were significantly higher in South Asians compared to Caucasians (12± vs. 11± pmol/L; p=0.01).

1.41.1.9 Stimulated levels

OGTT

Time courses (individual subjects) for plasma GLP-1 concentrations in South Asians and Caucasians are illustrated below (figure 5.14 & 5.15).

Figure 0.14 Individual total GLP-1 responses in South Asians during 50-gm OGTT

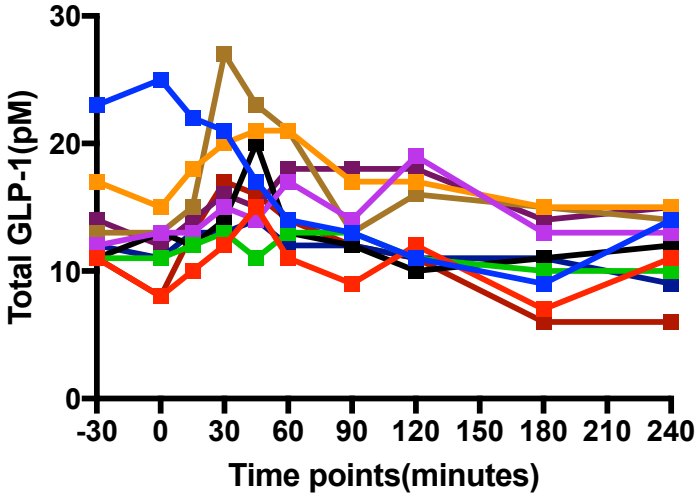
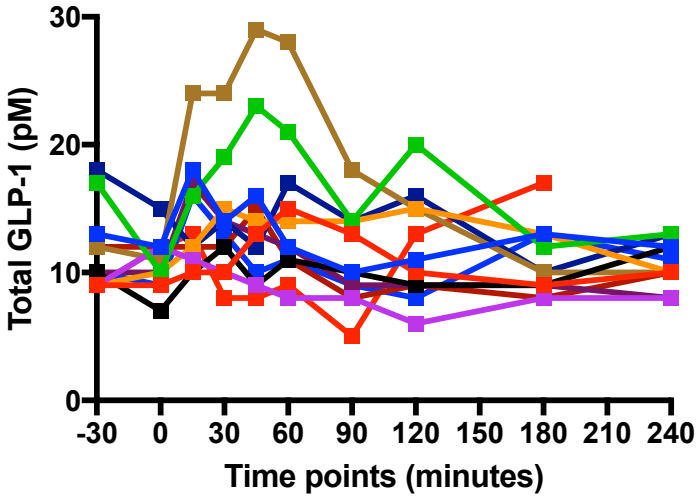


Figure 0.15 Individual total GLP-1 responses in Caucasian subjects during 50-gm OGTT.



IGII

Time courses (individual subjects) for plasma GLP-1 concentrations in South Asians and Caucasians are illustrated in figures below (figure 5.16 & 5.17).

In comparison to OGTT, there were no significant increase in GLP-1 secretory response during IGII.

Figure 0.16 Individual total GLP-1 responses in South Asian subjects during IGII.

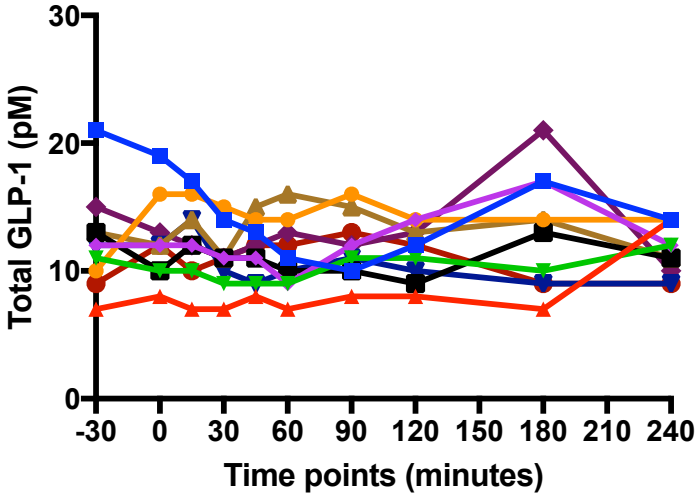
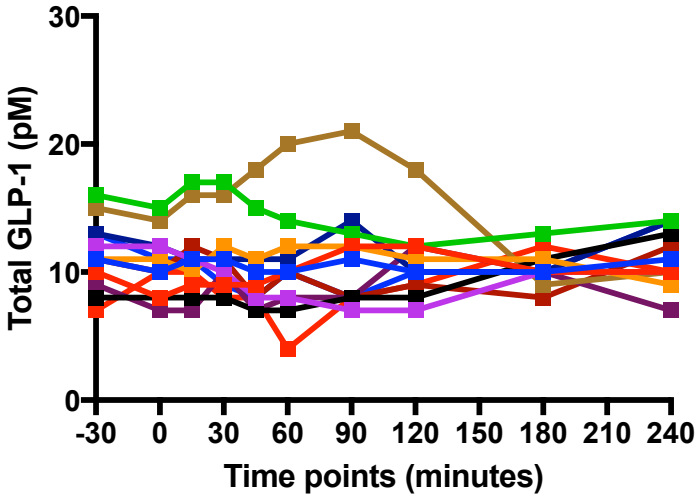


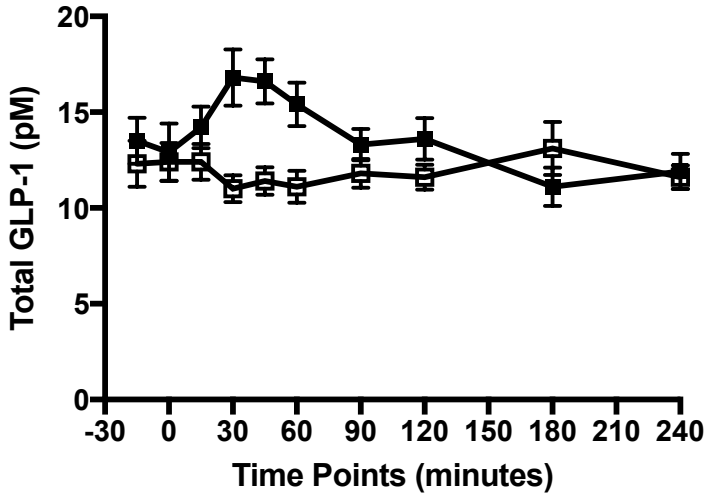
Figure 0.17 Individual total GLP-1 responses in Caucasian subjects during IGII.



Within groups (OGTT vs. IGII)

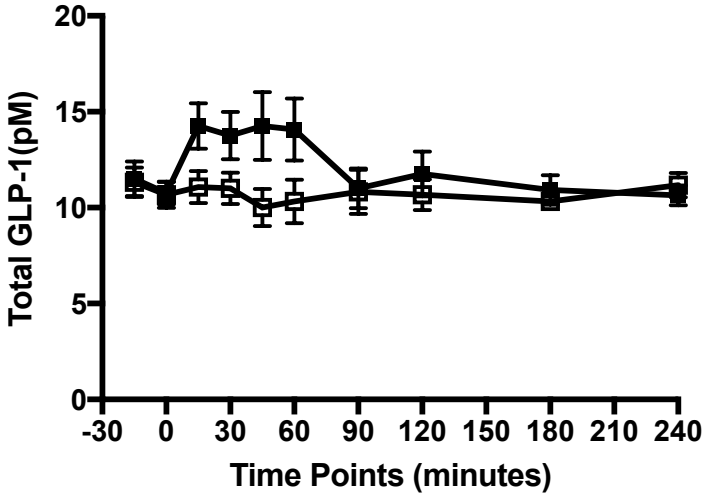
In South Asians, there was no significant difference in the total GLP-1 responses between OGTT and IGII (figure 5.18).

Figure 0.18 Total GLP-1 responses in South Asian subjects during 50-gm. OGTT(filled symbols) and IGII(open symbols).



In Caucasian subjects, there were no significant difference in total GLP-1 response between OGTT and IGII (2612 vs. 2469 pmol/l x min, p=0.2) (figure 5.19).

Figure 0.19 Total GLP-1 responses in Caucasian subjects during OGTT (filled symbols) and IGII (open symbols).

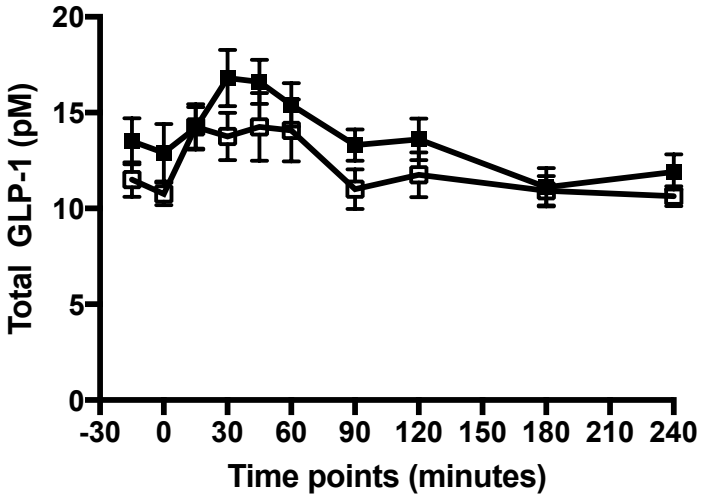


Between groups (South Asians OGTT vs. Caucasian OGTT)

Total GLP-1 responses following oral glucose ingestion, was similar (non-significantly higher) in South Asians compared to Caucasians (SA tAUC $_{OGTT}$ 3194±198 vs. CA tAUC $_{OGTT}$ 2840±181; p=0.20).

The difference in total AUC during OGTT and IGII, was also similar (non-significantly higher) in South Asians compared to Caucasians (SA tAUC $_{OGTT}$ - $tAUC_{IGII}$: 319 ± 86 vs. CA $tAUC_{OGTT}$ - $tAUC_{IGII}$: 284 ± 67; p=0.7).

Figure 0.20 Total GLP-1 responses during 50-gm OGTT in South Asian (filled symbols) and Caucasian subjects (open symbols).



Glucose-dependent Insulinotropic Polypeptide

1.41.1.10 Fasting

Mean fasting levels (OGTT and IGII) were similar in South Asians and Caucasian subjects.

OGTT

Time courses for plasma GIP concentrations in South Asians and Caucasians are illustrated in figures below (figures 5.21 & 5.22).

Figure 0.21 Individual GIP responses in South Asian subjects during 50-gm. OGTT

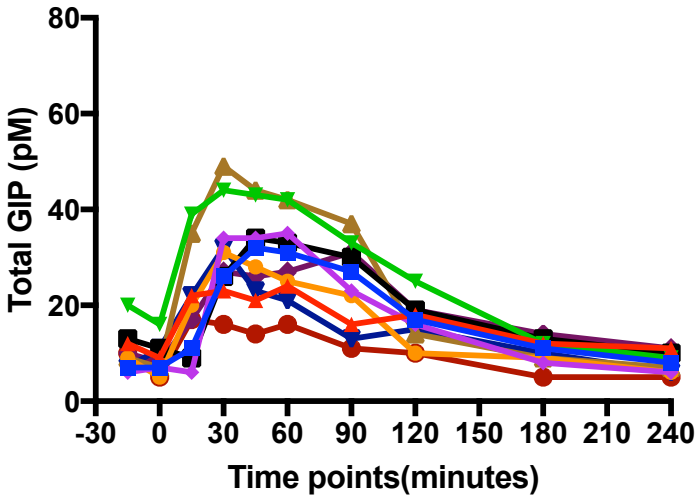
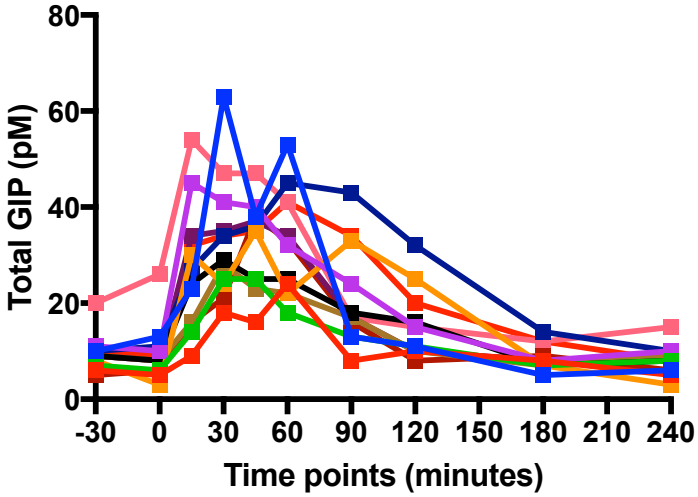


Figure 0.22 Individual GIP responses in Caucasian subjects during 50-gm. OGTT

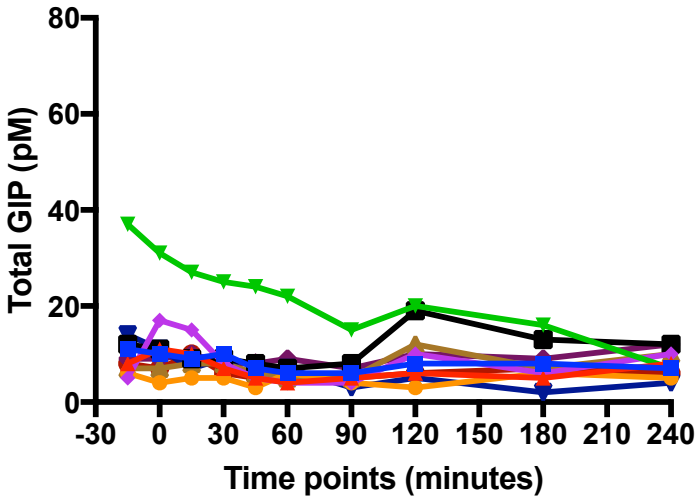


IGII

Time courses for plasma GIP concentrations in South Asians and Caucasians are illustrated in figures below (figures 5.23 and 5.24).

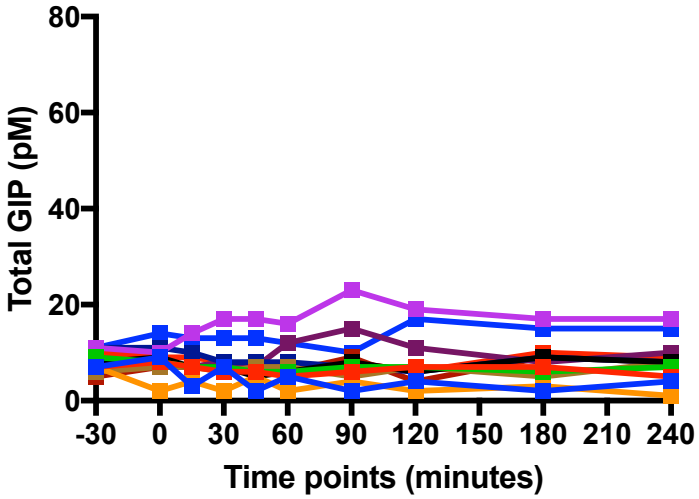
No significant GIP secretory response was detected following intravenous glucose infusion (IGII) in both groups.

Figure 0.23 Individual GIP responses in South Asian subjects during IGII



As noted in the above figure, one of the subjects (ID20SA) had much higher total GIP levels at baseline. This pattern was not noted during experiment one (OGTT).

Figure 0.24 Individual GLP-1 responses in Caucasians during IGII.



Within groups (OGTT vs. IGII)

Integrated GIP responses (tAUC) were significantly higher during OGTT in comparison to IGII, in both South Asians and Caucasian subjects
(SA $tAUC_{OGTT}$ 4889±404 vs. $tAUC_{IGII}$ 1829, p=0.0002; CA t AUC_{OGTT} 4814±379 vs. $tAUC_{IGII}$ 1956±287, p<0.0001).

Figure 0.25 Total GIP responses in South Asian subjects during 50-gm. OGTT (filled symbols) and IGII (open symbols).

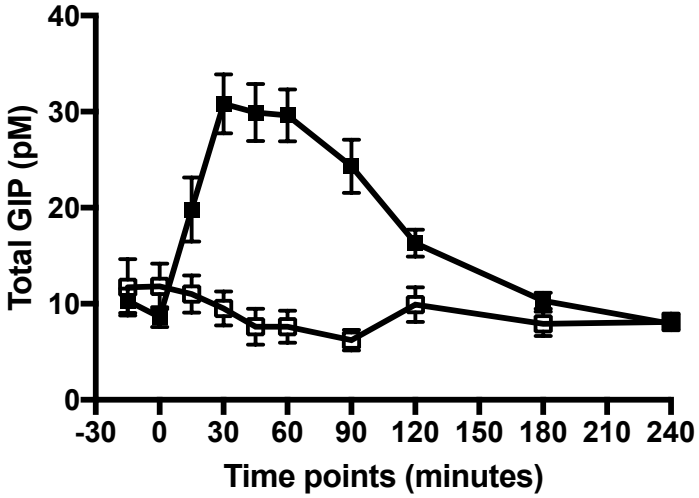
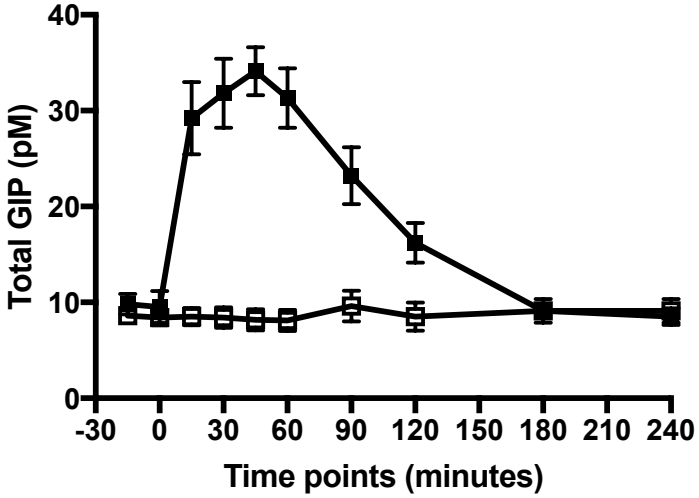


Figure 0.26 Total GIP responses in Caucasian subjects during OGTT (filled symbols) and IGII (open symbols).



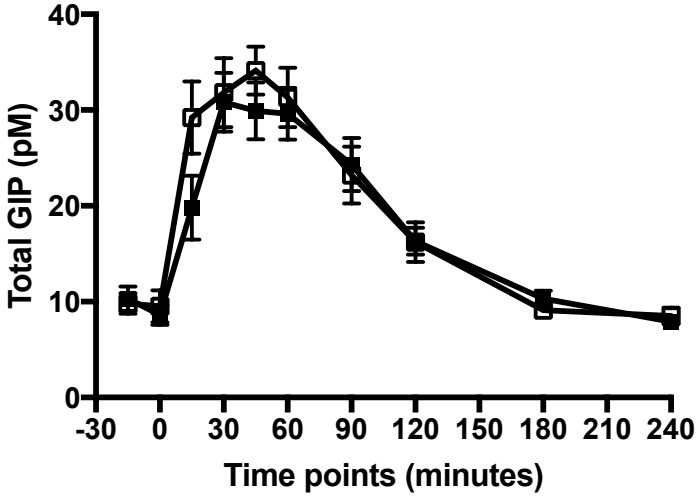
Between groups (South Asians vs. Caucasians)

Integrated total GIP responses (tAUC) during OGTT were similar amongst South Asians in comparison to Caucasians (SA t AUC_{OGTT} 4889±404 vs. CA t AUC_{OGTT} 4814±379; p=0.89).

Incremental responses (baseline subtracted) of total GIP during OGTT were significantly higher in South Asians in comparison to Caucasians (1887 vs. (minus) 273; p<0.0001).

The difference in total AUC between OGTT and IGII, was also similar in South Asians compared to Caucasians (SA $tAUC_{OGTT}$ - $tAUC_{IGII}$: 2233 ±220 vs. CA $tAUC_{OGTT}$ - $tAUC_{IGII}$: 2323±357 p=0.8)

Figure 0.27 Total GLP-1 responses during 50-gm. OGTT in South Asian (filled symbols) and Caucasian (open symbols) subjects.



Incretin effect

This was calculated using various surrogate measures of beta cell secretory function. Incretin effect was measured using the following formula based on beta cell secretory responses (AUCs): $100x(AUC_{OGTT}- AUC_{IGII})/AUC_{OGTT}$.

1.41.1.11 Gut mediated glucose disposal (GIGD)

No significant differences were detected in GIGD between South Asians and Caucasians (60.6±3.3 % vs. 58.8±4.4%, p=0.75).

Glucose consumption between the groups was also not significantly different during individual 15-minute intervals of the IGII study (Fig).

Though non-significant, South Asians appeared to have a prolonged glucose infusion requirement compared to Caucasians. This relates to our observation of prolonged duration of hyperglycaemia post oral glucose loading.

Glucagon

1.41.1.12 Fasting

Mean basal plasma glucagon levels were similar in South Asians and Caucasians (8 vs. 9 pmol/L; p=0.35).

1.41.1.13 Stimulated

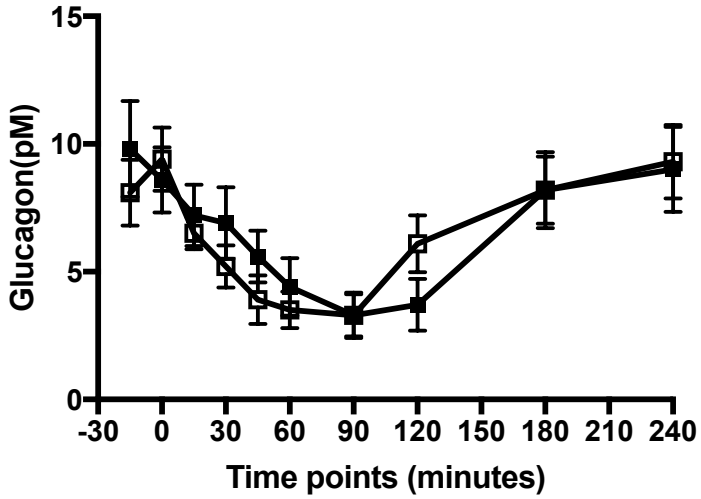
Within groups (OGTT vs. IGII)

South Asians

South Asians demonstrated a delayed suppression in glucagon levels during OGTT but an immediate suppression during IGII (nadir value at 60 minutes).

There was a delayed rise in the glucagon levels from nadir point, during OGTT in contrast a much brisker rise during IGII.

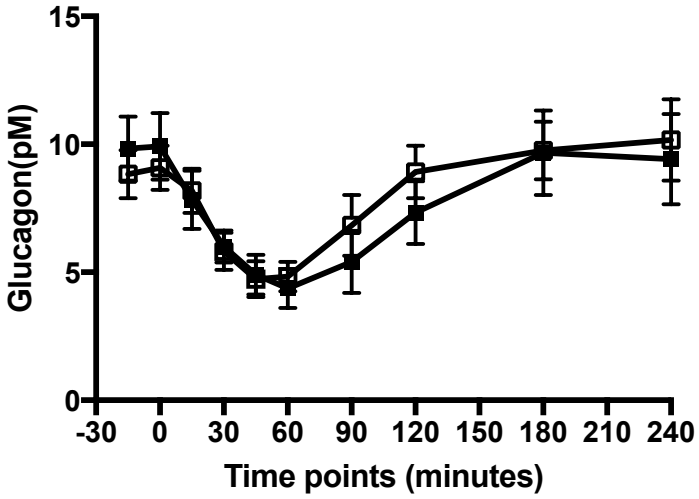
Figure 0.28 Glucagon responses in South Asian subjects during 50-gm. OGTT (filled symbols) and IGII (open symbols).



Caucasians

Plasma glucagon levels dropped abruptly post glucose ingestion as well following i.v. infusion of glucose, demonstrating equal suppression following OGTT and IGII.

Figure 0.29 Glucagon responses in Caucasian subjects during 50-gm. OGTT (filled symbols) and IGII (open symbols).



Between groups (South Asian vs. Caucasian)

The time points for nadir glucagon levels were 60 minutes in South Asians in comparison to 90 minutes in Caucasians, reflecting a brisker suppression amongst South Asians. Nadir glucagon levels were lower in South Asians (non-significant) compared to Caucasians. South Asians demonstrated a slower rise in glucagon levels following immediate suppression post glucose ingestion.

Figure 0.30 Glucagon responses during 50-gm. OGTT in South Asian (filled symbols) and Caucasian subjects.

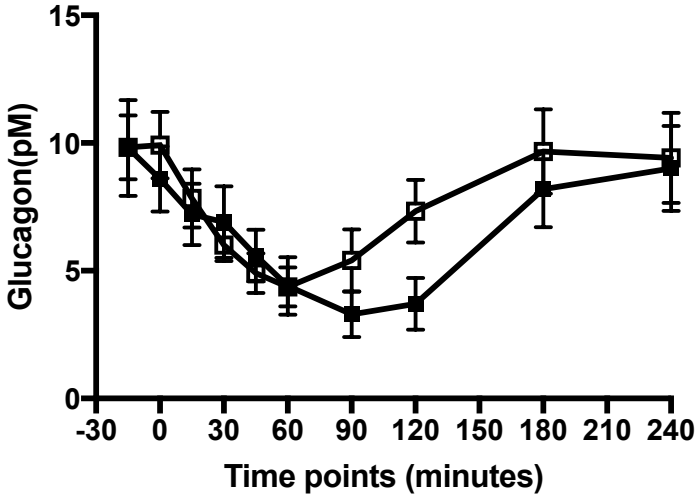


Table 0.2 Insulin, C-peptide, total GLP-1 , total GIP and glucagon responses

	South Asians N =10	Caucasians N=12	P value
Insulin			
Baseline $_{OGTT}$ pmol L^{-1}	11.9±1.6	7.7± 0.9	0.02
tAUC $_{OGTT}$ (240 min x pmol L^{-1})	10239±1683	5377±616	0.008
tAUC $_{IGII}$ (240 min x pmol L^{-1})	5610±1347	2703±321	0.03
P value $_{(tAUC\text{-}OGTT\ vs.\ IGII)}$	0.04	0.0009	
C-peptide			
Baseline $_{OGTT\&IGII}$ pmol L^{-1}	489.6±63.66	641.7±38.22	0.03
tAUC $_{OGTT}$ (240 min x pmol L^{-1})	352845±53954	350099±20144	0.95
iAUC $_{IGII}$ (240 min x pmol L^{-1})	86231±20082	75381±16678	0.68
Total GLP-1			
Baseline $_{OGTT}$ pmol L^{-1}	12(11-13.75) ^	10(9-18) ^	0.03*
Baseline $_{IGII}$ pmol L^{-1}	12(10-13) ^	11±0.5	0.27
P value $_{(OGTT\ vs.\ IGII)}$	0.20	0.13	
tAUC $_{OGTT}$ (240 min x pmol L^{-1})	3194±198	2840±181	0.20
tAUC $_{IGII}$ (240 min x pmol L^{-1})	2875±164	2556±132	0.14
P value $_{(OGTT\ vs.\ IGII)}$	0.23	0.21	
Total GIP			
Baseline $_{OGTT}$ pmol L^{-1}	8.5^	8.5^	0.78
Baseline $_{IGII}$ pmol L^{-1}	10^	8.37± 0.65	0.22
P value $_{OGTT\ vs.\ IGII}$	0.42	0.48	
tAUC $_{OGTT}$ (240 min x pmol L^{-1})	4889±404	4814±379	0.89^
tAUC $_{IGII}$ (240 min x pmol L^{-1})	1829	1956±287	0.92
P value $_{(OGTT\ vs.\ IGII)}$	0.0002^	<0.0001	
Glucagon			
Baseline $_{OGTT}$ pmol L^{-1}	9.2±1.6	9.8±1.2	
Baseline $_{IGII}$ pmol L^{-1}	8.7±1.2	8.9±0.8	
P value $_{(OGTT\ vs.\ IGII)}$			
tAUC $_{OGTT}$ (240 min x pmol L^{-1})	1491±252.4	1784±281	0.45
tAUC $_{IGII}$ (240 min x pmol L^{-1})	1523±232	1941±225	
P value (OGTT vs. IGII)	0.92	0.66	
Nadir glucagon $_{OGTT}$	2.3±0.73		
Nadir glucagon $_{IGII}$	2.7±0.7	3.9±0.7	0.22
P value $_{(OGTT\ vs.\ IGII)}$	0.69		

1.42 Discussion

Our findings demonstrate a similar incretin effect despite presence of insulin resistance and hyperinsulinaemia in normal glucose tolerant South Asians in comparison to a matched group of Caucasians.

The upregulation of insulin secondary to insulin resistance, as part of an adaptive response of maintaining glucose homeostasis, is well known (307).
The lack of 'up regulated' incretin effect despite insulin resistance, may suggest a lack of 'adaptive' incretin response in South Asians.
The evidence for potential up regulation of incretin hormones in response to insulin resistance is limited. A study by Hansen et al(274) demonstrated the impact of short-term insulin resistance with glucose intolerance on the incretin effect. A twelve- day intervention involving high dose steroids, high calorie diet and physical inactivity induced insulin resistance and glucose intolerance in young healthy men. The intervention demonstrated a reduction in the incretin effect (72 ±5 vs. 43± 7%, P = 0.002) with increased GIP response during OGTT.
Hansen et al (297) also looked at the insulinotropic effects of incretin hormones in comparison to glucose, in the presence of temporary insulin resistance. The authors studied glucose induced insulin responses to GIP and GLP-1 using hyperglycaemc clamp conditions (10 mmol/L) before and after inducing insulin resistance. The group demonstrated that while the insulin responses to glucose infusion were up regulated almost 3 folds in the presence of insulin resistance, the insulinotropic responses of GIP (1.8-fold) and GLP-1 (1.4-fold) were not significantly higher. The lack of amplified insulinotropic properties of GLP-1 and GIP were more pronounced in the late phase response (10-120 minutes). Hansen et al induced insulin resistance for the above studies, in healthy males using three different strategies including high calorie diet, sedentary lifestyle and high dose glucocorticoids. Therefore, while there are post interventions' impaired incretin responses demonstrated in this study, the role of each of these individual interventions, in influencing the altered incretin responses would be difficult to ascertain. The mechanisms of insulin resistance in South Asians would be dissimilar to the interventions used in this study. Therefore, it would be not possible to conclude that similar lack of amplification in the incretin hormone responses could be expected in South Asians.

The study group also excluded subjects with first degree relatives with type 2 diabetes. This was intentional to exclude individuals with any early signs of beta cell dysfunction as previously reported (308, 309).
There are studies showing altered incretin system in the presence of hyperinsulinaemia in individuals with impaired glucose tolerance (169). However similar impairment in the incretin system was not noted in other high-risk groups.

First degree relatives of people with type 2 diabetes

While there is evidence of an early insulin secretory defect in this group, the size of the incretin effect and GLP-1 and GIP responses following oral glucose, remains comparable (182). Also, the insulinotropic effects of GIP were comparable under euglycaemic conditions (310). While a previous study did show impaired GIP responses in first degree relatives, under hyperglycaemic conditions (305), further evaluation by the group suggested an insulin secretory defect underpinning this impaired response rather than an inherent GIP defect.

Lack of up regulation

Another probable explanation for a lack of 'adaptive' or up-regulated incretin response maybe due to a less potent gut stimulation. As we know following a glucose load of 50 gram, the peak plasma glucose level achieved in South Asians was 7.7 mmol/L. This is not significantly higher than a pre meal glucose range (4-7 mmol/L).

While the actual glucose stimulated incretin hormone responses in our study (total GIP and GLP-1 levels) are comparable between Caucasians and South Asians, overall South Asians appear to have a non-significantly lower level of glucose stimulated GLP-1 levels, in comparison.

It also appears that the glucose stimulated GLP-1 response was prolonged in South Asians compared to Caucasians.
In our study there were no significant difference in the tAUC values of GLP1 between South Asians and Caucasians. This is consistent with the study reporting impact of obesity on incretin hormone responses (255). This study included lean and obese subjects with and without diabetes.

The findings from our study could be interpreted in various ways.

The incretin effect is comparable in South Asians despite presence of insulin resistance. This may suggest that the mechanism of hyperinsulinaemia with underlying insulin resistance in South Asians is independent of the gut-mediated insulin secretion i.e. incretin response.

Another probability is that there is a degree of incretin resistance in South Asians similar to insulin resistance. This may be reflected by the fact that there is no further potentiation of incretin induced insulin secretion in the presence of endogenous insulin resistance. Incretin resistance similar to insulin resistance has previously been reported.

Chapter 6

Comparing post prandial Non-esterified fatty acids following meals

1.43 Plasma NEFA and physiology

Non-esterified fatty acids (NEFA) also called free fatty acids (FFA) are released on hydrolysis of triglycerides from adipose tissue. They are considered part of metabolic adaptability. Circulating FFA concentrations lower post meals as postprandial insulin production inhibits adipose tissue lipolysis. Conversely plasma FFA levels rise overnight following a fasting state.

Acute exposure to FFA is known to increase basal and glucose stimulated insulin secretion in lean subjects. This response in blunted in the presence of obesity. The acute enhancement of glucose stimulated insulin secretion appears to be related to the type of NEFA ingested with the effect more relevant with monounsaturated rather than polyunsaturated or saturated fats (311).

Reducing FFA levels has shown to increase glucose induced insulin secretion. FFA regulation promotes higher glucose excursions (312) through delayed glucose disposal(313) and increased endogenous glucose production (314).

1.44 Role of NEFA in insulin resistance & obesity

Plasma free fatty acids are increased in obesity particularly in case of visceral adiposity. High circulating FFA are associated with dysglycaemia and dyslipidaemia. They are also known to be an independent risk factor for cardiovascular disease.

NEFA in South Asians

The role of plasma NEFA in insulin resistance in normal glucose tolerant South Asians has been extensively explored.

Circulating NEFA results in increased insulin resistance at most target organs including skeletal muscles, liver and endothelial cells. South Asians have been noted to have reduced adiponectin and increased circulating NEFA levels associated with larger abdominal subcutaneous adipocytes compared to white European counterparts (315). For a given level of adiposity, South Asians have higher circulating levels of NEFA than matched Caucasian individuals (316).

The role of NEFA metabolism in mediating dysglycaemia in South Asians, has been previously noted (317, 318).

One of the proposed mechanisms for increased NEFA levels is related to altered sequestration of NEFA from blood and the role of translocase CD36 in South Asians (319).

1.45 Obesity in South Asians

Background

There are three type of adipose tissue namely superficial subcutaneous, deep subcutaneous and visceral fat. While the superficial subcutaneous fat is the primary component, it is also predominantly metabolically inert. The other components are related to high transmembrane fluxes and hence associated with dysglycaemia and dyslipidaemia.

Adiposity in South Asians

South Asians are known to have more adipose tissue mass at a given BMI in comparison to White European counterparts (77). In addition, there is presence of higher visceral adiposity despite similar BMI, amongst South Asians, in comparison to Caucasians (77). Overall body fat distribution in South Asians is also different with propensity for fat deposition in lower rather than upper body. When compared to Caucasians, peripheral subcutaneous fat is also less in South Asians. This has been noted even amongst young children, when comparing skin fold thickness (78). The morphology of subcutaneous adipose tissue cells in South Asians is also believed to be different compared to Caucasians, resulting in limited storage capacity (73, 80). South Asians have higher levels of liver fat compared to White Europeans (73) (74).
Varied body fat distribution has been proposed as one of the plausible factors underpinning increased risk of cardiovascular disease and diabetes amongst Indians (320).

Some of the proposed reasons for altered fat distribution with increased obesity in South Asians are as follows

1.45.1.1 Thrifty genotype

This was first proposed by JV Neel in 1962 (81). He postulated that certain individuals are programmed with a lower threshold to store excess energy to survive potential episodes of food scarcity such as famines. However, this is counterproductive when the same group is exposed to continuous periods of excess nutrition as it results in early onset obesity with other downstream risks including diabetes. This theory has been extensively debated in recent times. The current view is now in favour of the fact that the thrifty gene theory confers an advantage for procreation rather than survival per se.

1.45.1.2 Low birth weight/thrifty phenotype

Hales & Barker proposed that individuals with low birth weight were at higher risk of diabetes, dyslipidaemia and vascular disease (321). Lower birth weight, a marker of fetal malnutrition, is more prevalent amongst babies born to South Asian women. More recent understanding suggests that the overall risk of adult onset diabetes is related to the 'catch-up' weight gain that occurs following birth, rather than the actual birth weight per se(83). In addition low birth is also prevalent amongst children born to South Asian mothers in the United Kingdom making malnutrition as unlikely causal factor (84, 85).

1.45.1.3 Adipose tissue overflow hypothesis

This was proposed as one of the mechanisms driving increased insulin resistance at a lower BMI in South Asians (86). This is a more recent hypothesis that proposes a relatively smaller capacity amongst South Asians, to deposit adipose tissue in superficial compartments. As a result, these sites when saturated as a result of increasing adiposity, leads to further adipose tissue deposits in secondary compartments such as subcutaneous and visceral compartments. The deep adipose tissue compartments are more metabolically active with increased transmembrane free fatty acid fluxes resulting in dysglycaemia and dyslipidaemia (86).

1.45.1.4 Drifty genotype

The theory suggests that, in the absence of predation selection pressure, genes that promote energy storage and obesity were not removed by natural selection and simply were allowed to drift in the genetic journey of human evolution, such that they explain the obesity pandemic in modern western societies. The drifty genotype theory was proposed by Speakman, in contradiction to the previously known thrifty gene hypothesis (87).

1.46 NEFA and incretins

Fatty acids are known to mediate physiological responses by activating G protein coupled receptors (GPR) particularly GPR41. These transmembrane receptors are extensively expressed in pancreatic beta cell and insulin producing cell lines including human islets and also in the ileum, monocytes, pancreatic α-cells, enteroendocrine cells, breast cancer cells, osteoclasts and some areas of the human brain (322).
Given their expression in enteroendocrine cells, it has been hypothesised that plasma FFA levels may influence the incretin hormone dynamics.
Recent in vitro studies on human islets have suggested that elevated FFA levels may impair incretin function (323, 324) . However murine studies have shown a lack of lipid induced beta cell impairment in the presence of diabetes (325). Another group has demonstrated lack of changes in GLP-1 and GIP levels, despite deterioration in glucose tolerance following one week of high fat diet in healthy individuals (326).
A much more recent proof of concept study interrogated the bidirectional effects of plasma NEFA levels on incretins in both healthy individuals and those with type 2 diabetes (327). Firstly, in healthy individuals, the group studied the effects of acute elevation of FFA levels on incretin mediated insulin secretion, by using a lipid infusion. Secondly in the diabetes subjects, the investigators looked at the incretin effect following acute suppression of NEFA levels using acipinox. While elevated NEFA levels, did impair incretin induced insulin potentiation in healthy individuals, acutely lowering the levels did not result in any improvement of incretin effect in those with type 2 diabetes. The authors concluded that NEFA levels appear to have a unidirectional impact on incretin effect.

There are some other studies looking at effects of raised lipid levels on the dynamics of glucose regulation in healthy and 'at risk' population. Kashyap et al showed a marked improvement in first phase insulin response in healthy individuals after 4 days of lipid infusion but an opposite response in those with family history of type 2 diabetes (328). On the other hand, another similar study showed an improvement in acute insulin response to intravenous glucose following 48 hours of acipimox, in subjects with family history of type 2 diabetes (329).

At this stage it's important to note that reversibility of incretin effect may not be demonstrable in individuals with established type 2 diabetes.

Incretin and NEFA in South Asians

To the best of our knowledge, we report the first ever study looking at the relationship of incretins with FFA in South Asians. Given the design of our study we have compared FFA responses between South Asians and Caucasians post meals and also during OGTT and isoglycaemic infusion studies.

1.47 Subjects & methods

As described in chapter four.

Adjusted NEFA

For any significant differences in the post-prandial NEFA response observed between both South Asians and Caucasians, the post-prandial change in NEFA adjusted for circulating C-peptide, which reflects the response in plasma NEFA within the ethnic group following adjustment for C-peptide, was calculated using the following equation:

$$Adjusted\ NEFA = \frac{\text{-}(NEFA_{baseline} - NEFA_{nadir})}{(C-Peptide_{peak} - C-Peptide_{baseline})}$$

1.48 Results

For the purpose of this chapter we will focus on the results of plasma FFA. Other parameters have been discussed at length in chapter four.
Eleven NGT South Asian (5 women; mean ± SEM age: 35±4 years; BMI: 24.7±1.0 kg/m^2; fasting plasma glucose (FPG): 4.7±0.2 mmol/L) and 15 Caucasians with similar age and BMI
(8 women; age: 32±3 years; BMI: 25.1±1.0 kg/m^2; FPG: 4.6±0.1 mmol/L) consumed three isocaloric liquid meals (~500 kcal) of varying compositions (carbohydrate-rich (CHO), mixed (MIX) and fat-rich (FAT)).

Both fasting NEFA and insulin levels were higher in South Asians compared to Caucasians (NEFA: 0.60±0.02 vs.o.47± 0.02 mmmol/L, p =0.003; Insulin: 11.02±0.9 vs.7.3±0.4 mU/l, p<0.0001). Insulin responses (area under the curve (AUC)) were higher in South Asians compared to Caucasians (CHO: 27,631±5901 vs. 10,352±900 mU/l×min, *P*<0.0001; MIX: 15,548±3295 vs. 8,064±873 mU/l×min, *P*=0.02; FAT: 7,228±1,092 vs. 4,027±438 mU/l ×min, *P*=0.006).

Postprandial NEFA responses (AUC) were lower in South Asians compared to Caucasians (CHO: 3.8±1.5 vs. 5.5±2.1 mmol/L×min, *P*=0.002; MIX: 7.5±1.5 vs. 10.0±1.5 mmol/L×min, *P*=0.2; FAT: 18.64±2.1 vs. 20.53±2.2 mmol/L ×min, *P*=0.5).

The post-prandial NEFA suppression for all three meals was shown to be less in South Asians compared to Caucasians. However, South Asians were shown to have a significantly lower NEFA suppression following the CHO meal than Caucasians
(-3.8±1.5 vs. -5.5±2.1 mmol/l x min, p=0.0025).

1.49 Discussion

Normal glucose tolerant South Asians compared to Caucasians, had significantly higher fasting NEFA levels. Following a carbohydrate-rich meal, reduced postprandial suppression of NEFA levels was evident in South Asians compared to Caucasians. However postprandial NEFA suppression was comparable following mixed and fat-rich meals. After determining the adjusted NEFA levels which took into account differences in circulating insulin levels between both cohorts, NEFA suppression during carbohydrate-rich meals were actually similar between both groups. Therefore, overall postprandial suppression of NEFA levels were comparable between both groups.

The relationship between circulating levels of NEFA and incretin hormones (incretin effect and post prandial responses) is yet to be explored.

Chapter 7

Summary discussion, limitations, future directions and conclusions

Summary discussion, limitations, future directions and conclusions

1.50 Summary discussion

Insulin responses, sensitivity and secretion rates

Despite comparable fasting glucose levels, South Asian subjects demonstrated reduced insulin sensitivity as reflected by the Matsuda index, a composite marker of peripheral and hepatic insulin sensitivity. South Asian subjects also demonstrated a higher incremental insulin response with higher insulin secretin rates (ISR) to different meal stimuli, in comparison to Caucasian subjects. These differences were more pronounced in the second rather than the first phase insulin response.

Incretin Hormones (total GLP-1 and GIP)

1.50.1.1 Fasting levels

Baseline (fasting) total GLP-1 and GIP levels were significantly higher in South Asians compared to Caucasians. This is concordant with previously reported findings of raised basal incretin hormone levels in the presence of insulin resistance.

1.50.1.2 Meal responses

Fasting GLP-1 levels were higher in South Asians. However, unlike the insulin response in the presence of insulin resistance, there was a lack of augmented GLP-1 response post meals. Fasting GIP levels were comparable between both groups. However, the total GIP responses were higher (non-significant) amongst South Asians subjects. Higher GIP response in insulin resistant South Asians is similar to previously reported finding of exaggerated GIP response in the presence of glucocorticoid induced insulin resistance. Therefore, despite significantly higher fasting total GLP-1 levels, South Asian subjects did not demonstrate an adaptive incretin response following isocaloric meals with varying nutrient stimuli. This raises the possibility of hyperinsulinaemia in the presence of insulin resistance in NGT South Asians, occurring independent of an augmented incretin response.

On the other hand, lack of an augmented GLP-1 response post meal in South Asians may be due to an impaired adaptive incretin response in this group.
The role of varying gastric emptying potentially influencing GLP-1 responses remains to be explored.

1.50.1.3 Incretin effect

Despite presence of insulin resistance and hyperinsulinaemia, South Asian subjects demonstrated a similar incretin effect compared to Caucasians with similar age and BMI.
In our study South Asian subjects appear to have non-significantly lower level of glucose stimulated GLP-1 levels, in comparison to Caucasians. Glucose stimulated total GLP-1 responses were prolonged in South Asians compared to Caucasians suggesting the possibility of altered DPP - 4 activity. In terms of GIP, the mean fasting levels were similar between South Asians and Caucasians.

This draws our attention to the interplay between the incretin system and mechanisms resulting in endogenous insulin resistance in South Asians. Comparable incretin effect in insulin resistant but normal glucose tolerant South Asians and Caucasians potentially suggest that mechanism of hyperinsulinaemia with underlying insulin resistance in South Asians is independent of the gut-mediated insulin secretion i.e. incretin response. Our study demonstrated a lack of upregulation of the incretin effect despite presence of insulin resistance and hyperinsulinaemia in South Asians in comparison to Caucasians. Therefore, this also raises the possibility of incretin resistance similar to insulin resistance, accounting for a lack of further upregulation of the incretin hormone responses amongst insulin resistant South Asians. While an impaired incretin effect in the presence of glucocorticoid induced insulin resistance has previously been demonstrated, a lack of a similar result in a study amongst South Asians with endogenous insulin resistance, highlights the discrepancy in the involved mechanistic pathways in both cases.

Gut mediated glucose disposal (GIGD)

No significant differences were detected in GIGD between South Asians and Caucasians. Glucose consumption between the groups were also not significantly different during individual 15-minute intervals of the IGII study. Though non-significant, South Asians appeared to have a prolonged glucose infusion requirement

compared to Caucasians. This is likely to be reflective of the underlying insulin resistance in South Asian subjects.

Glucagon

1.50.1.4 Fasting levels

These were comparable between both groups.

1.50.1.5 Glucagon responses during meals

Integrated glucagon responses were similar during carbohydrate-rich, mixed and fat-rich meals in South Asians and Caucasians.

1.50.1.6 Glucagon responses during 50-gm. OGTT and IGII

Fasting glucagon levels were comparable in South Asian and Caucasian subjects.
South Asians demonstrated a slower rise in glucagon levels following immediate suppression post 50- gram glucose ingestion.

1.51 Limitations

Sample size

This was a very small observational study; therefore, no clear mechanisms for the observations can be established. However, it does lend key insights into our evolving understanding of the incretin axis amongst individuals of South Asian descent.

Age ranges

The age range of included subjects was fairly broad.
It is well known that glucose tolerance declines with age leading to an increased prevalence of type 2 diabetes amongst the older population (330) .
One of the key contributory factors playing a role in age related abnormal glucose metabolism is beta cell dysfunction (331),(332).
One of the attributory factors resulting in age related beta cell dysfunction, in the absence of type 2 diabetes, is potentially an age related altered incretin axis (333).
The investigators in this study by Ranganath et al, compared incretin hormone responses amongst 6 young (age 23.2 ± 1.8 yr.) premenopausal and 6 older (67.5 ± 4.1 yr.) postmenopausal women following oral 100-gram carbohydrate load (333). The fasting glucose and GLP-1 levels were significantly higher in the older group ($p<0.04$ and 0.03 respectively) but mean fasting insulin and GIP concentrations were similar. Following carbohydrate load, both GIP and GLP-1 integrated responses were considerably higher amongst the older group (almost 2-fold for GIP and 3-fold for GLP-1). The authors concluded that their findings demonstrated an impact of ageing on the enteroinsular axis as well provided further evidence for presence of negative feedback relation between incretin hormones and insulin secretion (333).

More recent studies looking at effect of ageing on various aspects of the incretin axis:

- **Trahair 2012 (334)**- Investigators in this study compared effects on glycaemic, insulinemic and incretin responses in 12 healthy young (six male, six female; age 22.2 ± 2.3 yr) and 12 older (six male, six female; age 68.7 ± 1 yr.) subjects following varying intraduodenal glucose load infusions. At baseline while insulin and glucose levels were higher in older subjects ($p<0.001$), GLP-1 and GIP levels were comparable between both groups. The study concluded that when glucose was infused into the small intestine at equal rates in young and older subjects, while glycaemic responses were greater in older subjects ($p<0.001$) both GLP-1 and GIP responses are comparable between groups.

- **Garduno-Garcia 2018 (335)**

In this recent study, incretin hormone secretion was compared in lean older subjects with either normal or impaired glucose tolerance with young lean subjects with normal glucose tolerance.

A total of 40 young (25 ± 3 yr.) and 53 older (74 ± 7 yr.) lean non-diabetic subjects underwent a two-hour glucose tolerance test. Based on the glucose tolerance, they were further divided into 3 groups (young with normal glucose tolerance; n=40, older subjects with normal glucose tolerance; n=32 and older subjects with impaired glucose tolerance; n=21). The investigators noted that the incretin response in older subjects was exaggerated rather than impaired amongst both normal and impaired glucose tolerant older subjects compared to the young. The authors concluded that the higher GLP-1 response amongst older subjects was suggestive of resistance of beta cells to the incretin effect and thus the gut may respond to a comparable glucose load with higher incretin hormone levels to achieve similar insulin responses.

Therefore, based on the evidence of the influence of ageing on the incretin hormone responses, having subjects of a wide age range within the study may have acted as a potential confounding variable.

Positive family history for type 2 diabetes

I did not match subjects based on positive family history within both the South Asian and Caucasian groups.
The incretin hormone responses are known to be influenced by a positive family history of type 2 diabetes.

Heritability of GLP-1 response to OGTT has been demonstrated by a study involving 35 monozygotic and 75 dizygotic twin pairs (336).
The authors also noted that an acquired unhealthy pattern of obesity associated with liver fat accumulation and insulin resistance is closely related to an impaired GLP-1 response in younger adults.
Hence, positive family history of type 2 diabetes and associated metabolic changes amongst some subjects, would again be a potential confounder in the study.

Smoking status

I did not match subjects based on their smoking status. In addition, there were no restrictions in terms of smoking as per the experiment protocol though patients did not smoke during the course of the individual experiment.

Both chronic as well as acute tobacco smoking have been shown to influence glucose homeostasis. The chronic effects of smoking resulting in increased insulin resistance include altered body fat distribution (337-339), increase inflammatory markers (340) and oxidative stress (341) as well as reduced glucose tolerance with reduced insulin secretion but minimal effects on fasting plasma glucose levels (342).

Acute effects of smoking also include impaired glucose tolerance and increase insulin levels during an OGTT (343). Also smoking is shown to worsen insulin resistance in patients with type 2 diabetes as demonstrated during an euglycaemic-hyperinsulinaemic clamp experiment (344). The acute effects of smoking on glucose homeostasis include reduced peripheral utilisation of glucose as a result of reduced insulin action (345) and altered post prandial glucose uptake from the gut by influencing gut and gall bladder motility (346, 347). Presence of nicotinic receptors on pancreatic alpha and beta cells also raises the possibility of a direct interaction as a result of both acute and chronic nicotine exposure and glucose metabolism (348).

A recent study from Denmark (349) has reported the effects of smoking on post prandial glucose homeostasis specifically focusing on GLP-1 and GIP levels, amongst heavy smokers without diabetes during a 4 hour mixed meal test in comparison to matched nonsmokers. In this study involving 11 subjects who were heavy smokers and 12 age-,sex- and BMI-matched nonsmokers, a 4-h liquid mixed- meal test was carried out with(immediately before and after meal intake) and without smoking in the case group and an identical meal test without smoking in the control group.
The investigators reported some interesting findings from the study.
The smoking group had significantly higher fasting glucagon levels along with exaggerated postprandial glucose changes when compared to matched to nonsmoking

controls. However, in contrast to the investigators' hypothesis as well some of the previous studies (343), smoking in conjunction with a mixed meal did not show a deterioration but rather an increase in postprandial glucose tolerance in comparison to nonsmoking test days. The improved post prandial glucose tolerance was associated with an unaltered insulin response and has been thought to secondary to smoking induced gastric emptying changes.

There was a lack of difference in fasting glucagon levels between smoking and nonsmoking tests days suggesting that this is likely an effect of chronic rather acute smoking.

The authors did not note a smoking induced direct effect on the postprandial plasma responses of GIP and GLP-1. This finding is concordant with other studies (350), (351).

Therefore, on reflection having included subjects without matching for smoking status may have been a potential confounder for my study.

Meal studies experiment

While all meals (carbohydrate -rich, mixed and fat-rich) were isovolaemic (350 mls.), the overall fluid consumption by each subject was not restricted. We did not limit the fluid intake during the experiment and the overall fluid consumption did vary amongst subjects. Therefore, the meals' studies were not strictly isovolaemic and hence may have had varying influence on gastric emptying for each subject. Access to free fluids maybe an important variable given its potential effect on gastric emptying and consequently on the incretin hormone responses.

Also, the meals were standardised in terms of compositions and calorie content for all subjects, but not matched for metabolic size. While subjects in both groups were similar in weight, they were not matched for metabolic size or estimated metabolic rate. It is well known that South Asians have higher visceral adiposity at lower BMI compared to Caucasian counterparts, which in turn influences their metabolic rate. Endogenous adiposity along with basal metabolic rate is known to influence post prandial insulin responses. Therefore, similar meal stimuli for all subjects not matched for metabolic size, could have potentially influenced our findings.

1.52 Future directions

Further mechanistic studies are required to improve our understanding of the islet incretin axis amongst people of South Asian descent. These studies need to include normal glucose tolerant South Asians as well as those with varying degrees of dysglycaemia leading up to type 2 diabetes.

1.53 Suggestions for future studies

- In terms of subjects, limit inclusion to either those originating from North or South India.

- Comparing incretin effect and meal responses in South Asians with impaired glucose tolerance would be important.

- To compare insulinotropic responses to placebo controlled supraphysiological doses of GLP-1 and GIP infusions in both South Asians and Caucasians.

Appendices

Appendix 1

EASD Abstract 2014

EASD 2014
541

Preserved incretin effect despite significant hyperinsulinaemia following glucose loading in South Asians in comparison to Caucasians

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INTRODUCTION

- Adaptive islet response results in up-regulation of insulin secretion in the presence of insulin resistance.
- Circulating hyperinsulinaemia in normal glucose tolerant(NGT) South Asians is reflective of underlying insulin resistance.
- Role of incretin hormones in this adaptive islet response in South Asians, remains to be explored.

STUDY AIM

Investigate the incretin effect in matched groups of normal glucose tolerant South Asians and Caucasians.

METHODS

SUBJECTS
Ten South Asian (age:33±4 yrs; BMI:24.5±1.1 kg/m2; fasting plasma glucose:4.8±0.2 mM) and twelve age and BMI-matched Caucasian subjects (age:31±3 yrs; BMI:24.6±1 kg/m2; fasting plasma glucose:4.6±0.1 mM) with normal glucose tolerance, were recruited.

STUDY PROTOCOL
All subjects underwent a 4hr paired oral glucose tolerance test (50 g) and isoglycaemic iv glucose (20%) infusion(IGII) study involving sampling for insulin, c-peptide, glucagon, GLP-1 and GIP, on 2 separate days. Blood glucose was measured using an YSI STAT analyser.

ANALYSES
All results were reported as means± SEM. The incretin effect was measured using the following formula based on beta cell secretory responses (AUCs): 100x(AUCOGTT- AUCIGII)/ AUCOGTT.

Table 1: Anthropometric data

	South Asians	Caucasians	p
N (F/M)	10 (4/6)	12 (7/5)	-
Age(yrs.)	34 (22-56)	31 (21-55)	NS
BMI	24.5 (19.4-31.6)	24.6 (19.6-35)	NS
Fasting plasma glucose(mM)	4.8 (4-5.8)	4.6 (4-5.1)	NS
75g- OGTT 2-hr glucose(mM)	4.8 (3-6.4)	3.8(2.2-5.8)	NS
Waist-hip ratio	0.85 (0.68-1.0)	0.83 (0.67-0.98)	NS

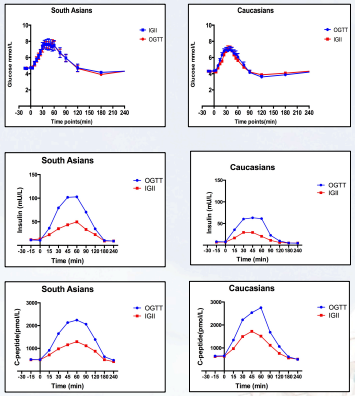

Figure 1: Glucose(top panel), insulin(middle) and c-peptide(lower panel) levels in NGT South Asians and Caucasians after 50-g OGTT and isoglycaemic intravenous glucose infusion.

RESULTS

- Fasting insulin levels were higher in South Asians vs. Caucasians (12.0±1.6 vs. 8.0±0.8 mU/l, p=0.02).
- No significant differences in fasting glucose levels on day 1(OGTT) and day 2(IGII) were observed within both groups.
- The oral glucose curves were replicated during the adjustable iv glucose infusions (with no significant differences between the two glucose curves in both groups)(top panel).
- The integrated insulin responses after OGTT and IIGI, respectively, were significantly higher in South Asians vs. Caucasians (OGTT: 10239±1683 vs. 5377±616 mU/l x min, p=0.008; IGII: 5610±1347 vs. 2703±321 mU/l x min, p=0.03).
- Incretin effects were similar in the two groups (48±6 vs. 49±2%, p=0.8).

CONCLUSION

- **Our findings show preserved incretin effect despite insulin resistance and hyperinsulinaemia in normal glucose tolerant South Asians.**
- **The role of incretin hormones in regulating glucose homeostasis in the presence of physiological euglycaemic hyperinsulinaemia in South Asians, remains to be studied.**

The study protocol(11/NW/0596) was approved by the National Research Ethics (NRES)- Liverpool Central North West committee. The study was conducted at the Clinical Research Facility in the Royal Liverpool & Broad green University Hospitals NHS trust in accordance with the principles of the Declaration of Helsinki.

1.54 Appendix 2

Late Breaking ADA abstract 2014

309-LB

Comparing Effects of Circulating Non-esterified Fatty Acids on Alpha and Beta Cell Responses Following Carbohydrate-rich, Mixed, and Fat-rich Liquid Meals between Normal Glucose Tolerant South Asians and Caucasians

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BACKGROUND

- Non-esterified fatty acids (NEFAs) stimulate endogenous insulin secretion along with mediating insulin resistance and pancreatic beta cell dysfunction
- Raised fasting NEFA levels and impaired insulin-mediated plasma NEFA suppression during oral glucose tolerance test, are often seen in South Asians

STUDY AIM

We aimed to compare fasting and postprandial levels of circulating NEFAs following meals of varying compositions, respectively, in normal glucose tolerant (NGT) South Asians and Caucasians.

METHODS

SUBJECTS
NGT South Asian (n=11) and age, gender and BMI-matched Caucasians (n=15) were studied (**Table 1**).

STUDY PROTOCOL
Following a 10h overnight fast all subjects consumed an isocaloric carbohydrate-rich (CHO), mixed (MIX) or fat-rich (FAT) liquid meal (**Table 2**) on three separate days.
Samples were drawn pre- and post meals for glucose, insulin, C-peptide and NEFA.

CALCULATIONS AND STATISTICS
Area under curve (AUC) was calculated using the trapezoidal rule.
Insulin secretion rate (ISR) values were calculated by the deconvolution method
The initial decrease of NEFA concentration following meals was calculated, for each subject, as ΔNEFA = $NEFA_{baseline}$ – $NEFA_{nadir}$.
The initial increase of C-peptide concentration was calculated as ΔC-peptide = C-$peptide_{peak}$ – C-$peptide_{baseline}$

TABLES & FIGURES

ANTHROPOMETRIC DATA (Table 1)

	SOUTH ASIANS	CAUCASIANS	p
N(M/F)	6/5	7/8	NS
Age	35 ± 3.7	32 ± 3	NS
Body Mass Index (kg/m²)	24.7 ± 1	25.1 ± 1	NS
Waist-Hip ratio	0.85 ± 0.02	0.85 ± 0.02	NS
Fasting glucose(mM)	4.7 ± 0.20	4.6 ± 0.10	NS
2- hour glucose(mM)	4.75 ± 0.30	3.9 ± 0.20	NS

LIQUID MEALS COMPOSITION (Table 2)

	CARBOHYDRATE-rich	MIXED	FAT-rich
CARBOHYDRATES: Kcal (total%)	419.42(83 %)	269.34(52.8%)	124.94(24.9%)
PROTEINS: Kcal(total%)	61.44(12.2%)	38.4(7.5%)	16.3(3.2%)
FAT: Kcal (total %)	22.5(4.5 %)	202.5(39.7%)	360(71.8%)
Total caloric content(Kcal)	503.35	510.15	501.1
Total volume(mls.)	350	350	350

C-peptide and NEFA levels during the three different isocaloric meals

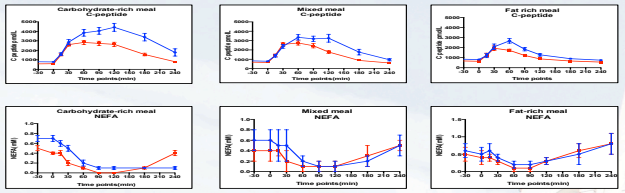

Figure 1: C-peptide (top panel) and NEFA levels in South Asians(blue) and Caucasians(red) measured during each of the three isocaloric meals.
Data points represent mean value± standard error of the mean.

RESULTS

- Both fasting NEFA (0.60±0.02 vs. 0.47±0.02 mM, P=0.003) and insulin levels (11.02±0.9 vs. 7.3±0.4 mU/l, P<0.0001) were higher in South Asians vs. Caucasians.
- Insulin responses (AUCs) were higher in South Asians vs. Caucasians during all three meal types (CHO: 27,631±5,901 vs. 10,352±900 mU/l×min, P<0.0001; MIX: 15,548±3,295 vs. 8,064±873 mU/l×min, P=0.02; FAT: 7,228±1,092 vs. 4,027±438 mU/l ×min, P=0.006).
- Pre hepatic insulin secretory responses (AUCs) calculated using the deconvolution method, were also higher in South Asians (CHO: 3,034±289 vs. 1,646±92 pmol/kg/min, p<0.0001;MIX: 1,980±217 vs. 1,250±67 pmol/kg/min, p=0.0002; FAT: 1,166±90 vs. 804±35 pmol/kg/min, p=0.0004).
- Postprandial NEFA responses (AUC) were lower in South Asians vs. Caucasians (CHO: 3.8±1.5 vs. 5.5±2.1 mM×min, P=0.002; MIX: 7.5±1.5 vs. 10.0±1.5 mM×min, P=0.2; FAT: 18.6±2.1 vs. 20.5±2.2 mM×min, P=0.5).
- The ratio ΔNEFA/ΔC-peptide was similar between both groups following each meal.

DISCUSSION

Normal glucose tolerant South Asians compared to Caucasians, demonstrate altered postprandial NEFA levels in the presence of higher insulin responses.
However, the NEFA suppression post meals were comparable between both groups. Overall, this may be reflective of increased insulin sensitivity of lipolysis in South Asians in comparison to Caucasians.
The potential role of incretin hormones in the dynamics of postprandial NEFA suppression in the presence of insulin resistance in NGT South Asians, remains to be studied.

The study protocol(11/NW/0596) was approved by the National Research Ethics (NRES)- Liverpool Central North West committee. The study was conducted at the Clinical Research Facility in the Royal Liverpool & Broad green University Hospitals NHS trust in accordance with the principles of the Declaration of Helsinki.

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