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ASSOCIATION OF CATESTATIN WITH INSULIN RESISTANCE DISPARITY IN NORMOGLYCAEMIC BLACK AND WHITE SOUTH AFRICAN WOMEN

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A dissertation submitted to the faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2024

Declaration

I Philisiwe Ntuli declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Ms Philisiwe Ntuli

On the ___09___day of ___July____2024

Dedication

I would like to dedicate this dissertation to my beloved parents, Mr MD Ntuli and Ms SS Ngubane. A special dedication to my husband Mr ZB Dlamini and my beautiful children Nobuhle, Philani and Ziphozenkosi.

Presentations arising from this study

Poster Presentations

Ntuli P, Snyman T and Toman M

Relationship of anthropometric measurements with indices of β-cell function and insulin resistance and clearance in healthy black African women, Pathology Research and Development Congress (PathRed), 31 August - 03 September 2023 (Johannesburg).

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Relationship between Chromogranin A, anthropometric indicators and surrogate indices of insulin regulation in apparently healthy African women, Society of Endocrinology Metabolism and Diabetes of South Africa (SEMDSA), 08-10 September 2023 (Johannesburg).

Ntuli P, Snyman T and Toman M

Relationship of anthropometric measurements with indices of β-cell function and insulin resistance and clearance in healthy black African women, Society of Endocrinology Metabolism and Diabetes of South Africa (SEMDSA), 08-10 September 2023 (Johannesburg).

Abstract

Background: Unexplained ethnic disparities exist in insulin resistance (IR) worldwide, affecting negatively more women of African ancestry than their white female counterparts. Insulin resistance is a pathological condition preceding the development of Type 2 diabetes (T2D), which in 2016 was the second leading cause of death in South Africa. The studies evaluating IR disparities in South Africa are very sparse, focussing mainly on obesity and fat distribution. However, IR has multifactorial aetiology and disparities have been reported irrespective of Body Mass Index (BMI) or age. Additional studies are therefore needed. From rodent-based and *in-vitro* studies it becomes clear that glycoprotein Chromogranin A (CgA) and its cleavage products catestatin (CST; found in pancreatic β-cells, having insulin-sensitising effects) and pancreastatin (PST; derived from pancreatic α-cells, having anti-insulin effects) are involved in the regulation of glucose metabolism.

We hypothesise that women of African ancestry may have low CST levels due to dysregulated CgA cleavage, yielding in an increased proportion of deregulatory PST. Therefore, we aim to determine whether circulatory levels of CST (or CgA; CgA/CST ratio) differ between healthy normoglycaemic black and white South African women independently of obesity and whether they are significantly involved in the regulation of IR.

Methods: "Apparently healthy" black (n=67) and white (n=54) women volunteers below 50 years of age from Gauteng province were enrolled in the study, following the enrolment criteria. Anthropometric measurements including weight, height, waist (WC) and hip (HC) circumferences and blood pressure (BP) were recorded. All participants underwent shortened oral glucose tolerance test (OGTT) to evaluate their glycaemic status except for externally enrolled white women (n=47). Samples were collected for measurements of glucose (fasting and 30-minutes), insulin (fasting and 30-minutes) and C-peptide (fasting) which were analysed by an automated Roche Cobas analyser. Other samples collected at fasting for CgA, CST, Interleukin 6 (IL-6), leptin, monocyte chemotactic protein-1 (MCP-1) and adiponectin were analysed by individual enzyme-linked immunosorbent assay (ELISA) kits. Surrogate indices for IR (HOMA-IR), early insulin secretion (Insulinogenic index; IGI), β-cell function (Disposition index; DIo) and hepatic insulin clearance (HIC) were calculated.

Indices of adiposity (BMI, waist to hip ratio (WHR), waist to height ratio (WHtR) and a body shape index (ABSI)) were calculated from obtained measurements. Demographic information and socio-economic status (SES) were evaluated by questionnaires.

Results: Black women were younger 26 (21; 32) than white women 30 (24; 35) and had higher BMI than white women (26.9 (22.4; 31.4) kg/m^2 vs 23.5 (20.8; 25.9) kg/m^2 respectively). Although normoglycaemic, white women had higher basal glucose levels (4.8 ± 0.3 mmol/L vs. 4.7 ± 0.3 mmol/L). No ethnic differences were found for fasting insulin or HOMA-IR. The only difference was attributable to adiposity in obese (OB) vs non-obese (NOB) black women. Similarly, no ethnic differences were observed for DIo and IGI. However, NOB black women had reduced ($p<0.001$) HIC in comparison with NOB white women. Black NOB women had reduced CST and elevated CgA (and CgA/CST ratio) than white NOB women, all with $p<0.001$. No associations of CST, CgA or CgA/CST were observed in white women. Catestatin correlated inversely with HIC ($p=0.045$) and positively with leptin ($p=0.010$) in black women. Correction for confounders removed this significance. CgA and the CgA/CST ratio correlated negatively with HOMA-IR and fasting insulin and positively with HIC. Only after removal of the strongest determinants, positive associations for CgA ($p<0.001$) and the ratio ($p=0.009$) remained in the model for HIC.

Conclusion: In the study population of normoglycaemic, normotensive and apparently healthy black and white women, we have not found elevated insulin levels or higher IR in the black ethnic group. However, we demonstrated for the first time the ethnic differences in CST with reduced levels and elevated levels of CgA and the CgA/CST ratio in black women vs white women. This difference is independent of obesity; we have not identified its reason but a genetic origin cannot be excluded. There were no direct regulatory effects of CST (CgA or CgA/CST) on IR. However, the study results indicate that HIC is the most closely associated variable of glucose-insulin dynamics with CST (CgA and CgA/CST ratio) in the black women in our study. The effects of CST on IR and glucose regulation may be indirect. The study found a positive correlation with leptin and CST-leptin interaction has been described previously in the literature**.** The binding of CST to insulin receptor results in reduction of obesity and this, in turn, can reduce IR and improve insulin and glucose levels**.**

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List of Abbreviations

ABSI	A Body Shape Index
ADA	American Diabetes Association
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BD	Becton Dickinson
BMI	Body Mass Index
BP	Blood Pressure
Ca^{2+}	Calcium Ion
Chi^2	Chi-squared (test)
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
COPD	Chronic Obstructive Pulmonary Disease
C-PEP	C-Peptide
CgA	Chromogranin A
CST	Catestatin
CV	Coefficient of Variation
CZ	Czech Republic, Europe
DI/DIo	Disposition Index (oral)
DIO	Diet-Induced Obesity
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay

ER	Endoplasmic Reticulum
FS IVGTT	Frequently Sampled Intravenous Glucose Tolerance Test
G-6-P	Glucose-6-Phosphate
GLC-F	Glucose fasting
GLUT-4	Glucose transporter 4
HC	Hip Circumference
HGP	Hepatic Glucose Production
HIC	Hepatic insulin clearance
HIV	Human Immunodeficiency Virus
HOMA (-IR)	Homeostatic Model Assessment (for Insulin Resistance)
HREC	Health Research Ethics Committee
HRP	Horseradish peroxidase
IFG	Impaired Fasting Glucose
IGF-1	Insulin-like Growth Factor 1
IGI	Insulinogenic Index
IGT	Impaired Glucose Tolerance
IL-6 (-8)	Interleukin-6 (-8)
IQR	Interquartile range
IR	insulin resistance
ISI	Insulin sensitivity index
IU	International units
KO	Knock Out
LEP	Leptin
LOD	Limit of detection

LOQ	Limit of quantification
MCP-1	monocyte chemoattractant protein-1
MDASLD	metabolic dysfunction-associated steatotic liver disease
MetS	Metabolic Syndrome
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate oxidised form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate reduced form
NAFLD	Non-alcoholic fatty liver disease
NHLS	National Health Laboratory Service
NOB	Non-obese
OB	Obese
OGTT	Oral glucose tolerance test
PST	Pancreastatin
QUICKI	Quantitative Insulin Sensitivity Check Index
RBP-4	Retinol Binding Protein 4
SAT	Subcutaneous adipose tissue
SD	Standard Deviation
SES	Socio economic status
SLE	Systemic Lupus Erythematosus
SOP	Standard Operating Procedure
SST	serum separating tube
TIMP-1	Tissue Inhibitor of Metalloproteinase 1
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor alpha
T2D	Type 2 diabetes

USA	United State of America
UV	Ultra Violet
VAT	Visceral adipose tissue
VIF	Variance inflation factor
Wits	University of the Witwatersrand
WC	Waist Circumference
WHO	World Health Organisation
WHR	Waist to Hip Ratio
WHtR	Waist to Height Ratio

Units and symbols

β	β
µ	micro
≤	less or equal
≥	greater or equal
%	percentage
α	alpha
mmol/L	millimoles per litre
pg	picogram
nmol	nanomole
µmol	micromole
cm	centimetre
m	metre
kg	kilogram
mmHg	millimetres of mercury

Chapter 1

1. Introduction - Literature Background

There are still unexplained worldwide-existing ethnic disparities in insulin resistance (IR). Women of African ancestry are more affected than African men (Goedecke et al., 2016) and their white female counterparts (Hasson et al., 2015).
The ethnic disparities in type 2 diabetes (T2D) are apparent already in the disease-associated risk factors (Wang et al., 2014; Yaghootkar et al., 2020) and from a young age such as in children (Arslanian et al., 2002) or adolescents (Arslanian et al., 1996).
It has been estimated that 1 in 8 adults (representing about 783 million people) will be affected with diabetes in 2045, which from 90% will be attributed to T2D. In South Africa, the all-over prevalence of T2D in individuals older than 15 years of age was 15.25% in 2020 (Pheiffer et al., 2021). The pathophysiology of T2D between Black Africans and their European counterparts may differ (Bergman et al., 2019; Goedecke and Mendham, 2022).

Insulin resistance is closely related to T2D and both share similar factors in their aetiology, although the exact cause of the disease is still not well known.
A strong component in the aetiology of IR is obesity (Kahn et al., 2006). However, the African-American (Haffner et al., 1996) and black South African (van der Merwe et al., 2000) women were more insulin resistant in Body Mass Index (BMI) and age-matched comparisons than their white counterparts. The disparities thus cannot be explained purely by obesity or differences in fat distribution (Van der Merwe et al., 2000, Goedecke[b] et al., 2009). Other mechanisms include e.g. inflammation, nutrition and socio-economic status (SES) or genetic background but outcomes of various studies differ (Hasson et al., 2015; Yaghootkar et al., 2020).

Numerous rodent-based and *in-vitro* studies have demonstrated that Chromogranin A (CgA; mostly known as a tumour marker) and some of its cleavage products are involved in the regulation of glucose metabolism, showing hormone insulin

sensitizing or insulin-opposing effects. Since information on these effects in humans is rather sparse, this study investigated possible involvement of CgA and, specifically, of its cleavage product catestatin (CST) in the above disparities in IR and associated steps of the glucose regulation.

In the following section of the Introduction Chapter, an overview on hormone insulin synthesis, secretion and dynamics in glucose regulation is given. The theories behind the roles of IR, β-cells insulin over-secretion and hepatic insulin clearance (HIC) in the pathophysiology of T2D are explained.

1.1 Insulin in glucose regulation

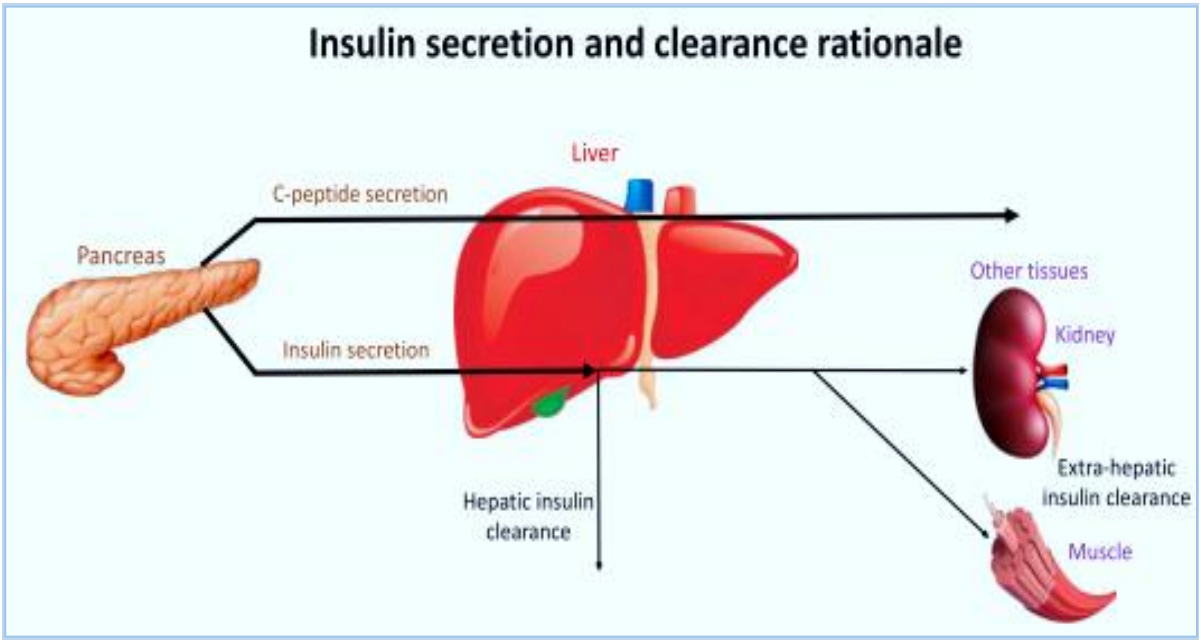
1.1.1 Insulin synthesis

Hormone insulin is synthesized in pancreatic β-cells from its precursor proinsulin. Proinsulin, after its formation from the pre-proinsulin in the endoplasmic reticulum of β-cells, is transported (together with other pro-hormones) to the Golgi complex. This is the site of glucose-regulated biogenesis of β-cell secretory granules. A specific sorting mechanism of a regulated secretory pathway separates proinsulin from proteins targeted elsewhere (to other secretory pathways), before being packaged into the secretory granule compartment. As a part of the granule maturation, proinsulin is enzymatically cleaved to insulin and C-peptide (Malaisse, 1997; Omar-Hmeadi, 2020).

Insulin secretion is either basal (post absorptive; between meals and during overnight fasting) or stimulated (postprandial; after food/glucose ingestion; regulates glucose disposal) (Del Prato et al., 2002). A specific metabolic trigger (e.g. high glucose levels) linked to calcium ions influx causes a co-release of the granule secretory products from their storage into the portal circulation by exocytosis. Insulin and C-peptide are co-secreted in equimolar amounts (Polonsky et al., 1983).

Glucose-stimulated insulin secretion occurs in a dose dependent manner. Thus, at fasting, insulin secretion levels are normally low. Due to the existence of two different

pools of the secretory granules, the stimulated insulin secretion follows a characteristic biphasic time pattern. The first phase (early phase) is short and transient with rapid insulin release. This enables quick inhibition of hepatic glucose production and increases glucose uptake by insulin-sensitive tissues after glucose/meal ingestion to restore normoglycaemia (Del Prato et al., 2002). Therefore, it has a major role in the postprandial glucose homeostasis. The second phase is developing slowly and is longer lasting.



Source: Piccinini and Bergman, 2020.

Figure 1.1: Insulin secretion and clearance rationale. Insulin and C-peptide are secreted in equimolar amounts from the pancreatic β-cells. Insulin is cleared in larger amounts while passing through liver, while C-peptide hepatic clearance is minimal.

Hepatic Insulin Clearance (HIC). From the portal circulation, secreted insulin and C-peptide enter liver where, to a different extent, both are degraded. Insulin concentration in the periphery thus depends on both insulin secretion from the β-cells (β-cell function) and the magnitude of HIC (Rubenstein et al 1972). Hepatic degradation of C-peptide is minimal (See **Figure 1.1**). Due to their equimolar secretion, C-peptide *per se* reflects the β-cells secretory function better than insulin

(Polonsky et al., 1983) and their molar ratio reflects the magnitude of insulin clearance by the liver.

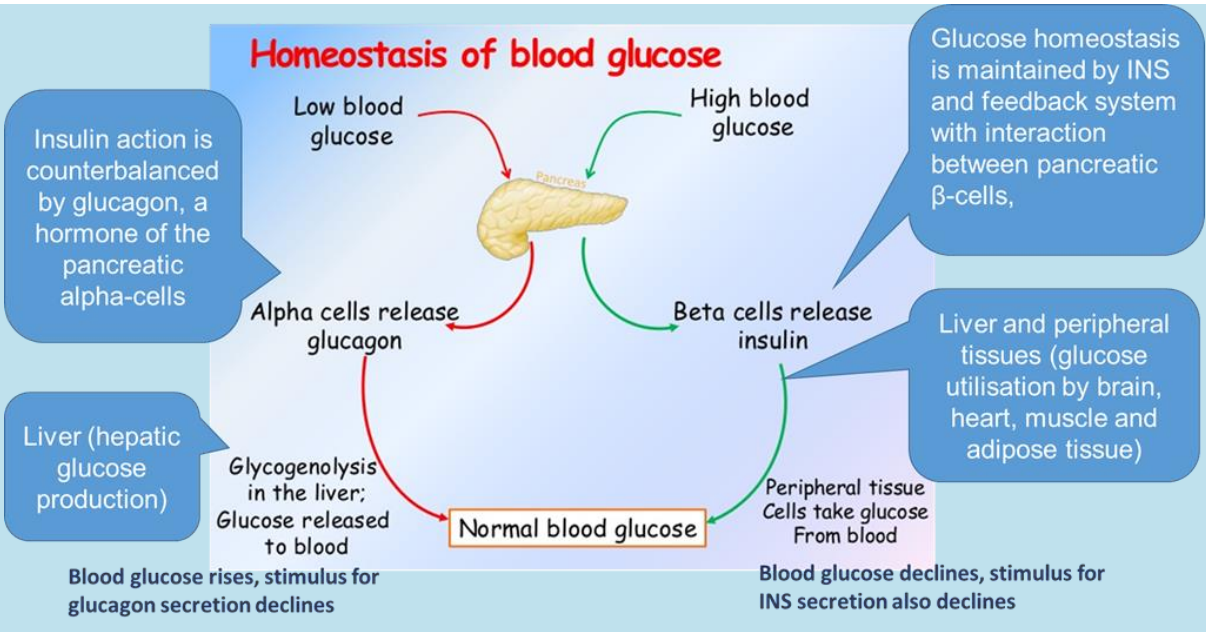
There is not much known about the molecular regulation of HIC, it may involve changes in insulin binding and cell signalling in response to external triggers such as dietary alterations (Bojsen-Møller et al., 2018). Total insulin clearance in the body includes extra-hepatic clearance by peripheral tissues such as kidney, muscle and adipose tissue (Polidori et al., 2016).

1.1.2 Pancreatic glucose regulation

Glucose homeostasis. Glucose is a main source of energy for many processes; its abnormal concentrations have impact on individual's survival (inadequately low levels; hypoglycaemia) or can lead to a development of metabolic degenerative diseases such as T2D (inappropriately elevated levels; hyperglycaemia). Normal glucose levels (euglycaemia; normoglycaemia) are regulated within relatively narrow physiological range irrespective of the energy intake or expenditure and they are essential for normal body's functions (Mann et al., 2020).

Glucose homeostasis is maintained by a closed feedback system involving the interaction between pancreatic β-cells (insulin secretion), liver (hepatic glucose production - HGP), and peripheral tissues (glucose utilisation by brain, heart, muscle and adipose tissue; Kahn et al., 2006).

Insulin and its counter-regulatory hormones. Insulin is the major hormone controlling metabolic processes in the fed state, reducing hyperglycaemia. However, its effects on a whole-body level are influenced by the interaction with other hormones. Insulin action is primarily counterbalanced by glucagon, a hormone of the pancreatic α-cells (Karam, 1997). **Figure 1.2** below summarises the glucose homeostasis and insulin action.



Adapted from Riaz, 2015

Figure 1.2: Glucose homeostasis and insulin action in maintaining normal blood glucose.

Insulin promotes uptake of blood glucose by target cells, liver, adipose and skeletal muscles to reduce blood glucose. It promotes glycolysis and glycogenesis and inhibits HGP. Glucagon reverses the above metabolic processes in order to increase blood glucose (promotes glycogenolysis, gluconeogenesis and ketogenesis).

Next to glucagon, insulin is counter-balanced by growth hormone, insulin-like growth factor 1 (IGF-1), glucocorticoids and catecholamines (epinephrine), which are released in response to elevated insulin to prevent hypoglycaemia (Rehman and Akash, 2016). Catecholamines promote lipolysis and glycogenolysis; glucocorticoids promote muscle catabolism, gluconeogenesis and lipolysis (Galicia-Garcia et al., 2020). Excessive secretion of insulin counter-regulatory hormones may contribute to IR in certain situations, but is not responsible for the great majority of IR conditions (Karam,1997).

1.1.3 Insulin action

Hormone insulin, after its release from pancreatic β-cells into circulation, stimulates glucose uptake and suppresses glucose production. Defects in both the availability and action of insulin lead to elevated plasma glucose levels and are major hallmarks of T2D (Omar-Hmeadi et al., 2020).

Insulin facilitates availability of energy sources so during fasting, energy is preserved, and during fed state, energy is utilised in order to maintain normoglycaemia (euglycaemia). In healthy individuals, in a fed (postprandial) state, insulin secretion from pancreatic β-cells increases as a response to adsorbed nutrients. Insulin reduces elevated blood glucose levels by various mechanisms:

1. Insulin promotes uptake of blood glucose into target cells (of liver, adipose, skeletal muscle) (Titchenell et al., 2017) to serve there as a metabolic fuel. For this action, insulin employs its receptor on the tissue cells plasma membrane. Insulin binding to this receptor triggers transmembrane signalling pathway that, in turn, modifies the intracellular metabolism. There are specific membrane transporters that facilitate glucose transport into the cells. Insulin signalling promotes transfer of these transporters from inside of the cell to the plasma membrane. Glucose transpoter-4 (GLUT 4) is the major glucose transporter in insulin-sensitive tissues (Weiss et al., 2014). Glucose uptake into muscle accounts for approximately 60-70% of total body mediated insulin uptake (Smith, 2002). Glucose uptake into the liver is not dependent on insulin, as the dietary carbohydrates are transported to the liver directly from the intestine (Adeva-Andany et al., 2016).
2. When glucose levels are high (fed state), increased insulin inhibits HGP (i.e. glycogenolysis and gluconeogenesis) (Titchenell et al., 2017) and, in turn, stimulates glycolysis (glucose conversion to pyruvate), and glycogenesis (stores glucose in a form of glycogen in the liver). This is supported by a decrease in HIC rate to keep insulin levels sufficiently elevated. In the fed state, insulin promotes glycogen synthesis through activation of glycogen synthase. This enables anaerobic energy release via glycolysis, e.g. during intensive muscle activity (Mann et al, 2020).

3. In fasting (basal; post absorptive i.e. between meals and during overnight fast) state, only low gluconeogenesis levels are maintained by insulin as essential supply of energy for glucose-dependent tissues (such as central and peripheral nervous system) (Del Prato et al., 2002; Mann et al., 2020). Muscle cells do not rely on glucose (or glycogen) for energy in the basal state. Low insulin levels (in starvation) reverse the above metabolic processes (Kahn et al, 1997) in order to normalise low glucose concentrations. The rate of HIC increases during fasting state to keep insulin at low (basal) levels (Mann et al., 2020).
4. Insulin acts on lipid and amino acid metabolism; in the postprandial state, intracellular glucose transport in adipocytes is insulin-dependent and facilitated by GLUT 4 (Adeva-Andany et al., 2016). Insulin controls the synthesis and storage of lipids in the liver (after glycogenesis saturation, the excess of glucose in the liver is utilised for fatty acid synthesis - lipogenesis) and stimulates accumulation of triglycerides in adipocytes. At the same time, insulin suppresses lipolysis and thus the flow of free fatty acids into the bloodstream. In insulin-deficient states, intracellular energy can be provided by fatty acid oxidation, while at the same time free fatty acids are released into the circulation for direct use by other organs.

   Insulin suppresses protein catabolism, while insulin deficiency promotes it, releasing amino acids for gluconeogenesis. In starvation state, protein synthesis is reduced by 50% (Giorgino et al., 2005).

1.1.4 Insulin sensitivity and insulin resistance

Insulin sensitivity is determined by the ability of the body's cells to respond to effects of insulin to lower the blood glucose. The decreased sensitivity (responsiveness) of target tissues to insulin leads to IR, which manifests as fasting hyperinsulinaemia and postprandial hyperglycaemia (Titchenell et al., 2017) and *vice versa*, high insulin sensitivity is associated with low IR. Therefore, they are inversely related. The whole-body IR includes hepatic and peripheral IR.

Glucose tolerance. Insulin secretion (β-cell function) and insulin sensitivity determine an individual's glucose tolerance and there is a hyperbolic relationship between them (Kahn et al., 1993). Their multiplication product is a constant, so-called disposition index (DI). It is used to evaluate β-cell function and the glucose-disposal ability of the body (Cobelli et al., 2007). To maintain normal glucose tolerance (normoglycaemia), healthy pancreatic β-cells compensate for IR (impaired insulin sensitivity) by increasing insulin secretion (Bergmann et al., 2002) as a feedback mechanism. This can manifest in different stages of glucose tolerance. As insulin sensitivity declines, so hyperinsulinaemia increases (Kahn et al., 2006). Insufficient compensation for IR results in impaired suppression of post-prandial glucose - impaired glucose tolerance (IGT).

1.1.5 Insulin resistance in the pathophysiology of T2D

Type 2 diabetes (mellitus) is a group of metabolic disorders characterized by hyperglycaemia (WHO, 2019). It has a multifactorial aetiology and pathophysiology. Next to defects in insulin sensitivity and secretion, this includes disturbances of carbohydrate, fat and protein metabolism. Individuals with T2D have missing the early phase of insulin secretion, which may play a role in the development of postprandial hyperglycaemia (Del Prato et al., 2002).

1.2 Pathogenesis of dysglycaemia and T2D

a) INSULIN RESISTANCE → HYPERINSULINAEMIA → T2D

Insulin resistance was considered a primary cause in the pathophysiology of T2D. This led to hyperinsulinaemia and subsequent failure of β-cells to compensate for the body's IR due to their "exhaustion". Resulting fasting and 2-hour hyperglycaemia, was then a later manifestation of T2D (Kahn et al, 2006).

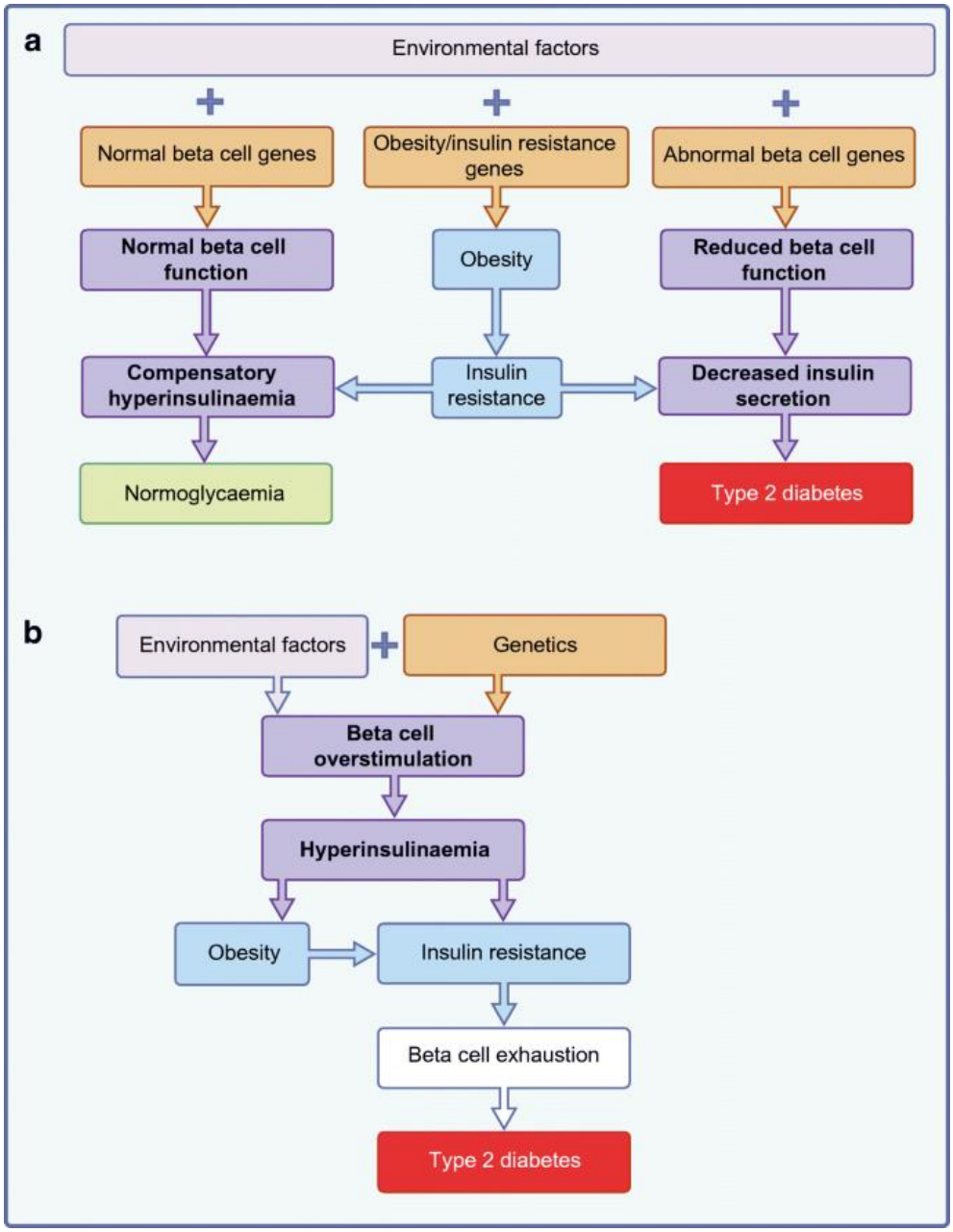
b) HYPERINSULINAEMIA DUE TO β-CELLS HYPERSECRETION → INSULIN RESISTANCE → T2D

However, a more recent theory suggests that insulin hypersecretion due to overstimulation of β-cells leading to their dysfunction is the primary independent event in the pathogenesis of IGT (Esser et al., 2020). This is associated with the development of obesity and IR as an adaptive response. Insulin-sensitive tissues, in order to maintain normal glucose tolerance, adjust to insulin hypersecretion by down-regulating their responsiveness (sensitivity) which will make them insulin resistant. Eventually, this will lead to hyperglycaemia, β-cells failure and T2D, **Figure 1.3** (Esser et al., 2020).

c) HYPERINSULINAEMIA DUE TO REDUCED HIC → INSULIN RESISTANCE → T2D

Still another pathway has been suggested by Bergman et al (2019), where the initial trigger is reduced HIC which could be heritable and/or influenced by the environment and nutrition. This would promote systemic persistent hyperinsulinaemia, IR followed by hyperglycaemia, β-cells failure, and ultimately T2D.

Thus IR, β-cell dysfunction and reduced HIC are involved in T2D pathogenesis. However, which is the primary trigger and what is the sequence of events it is still debated.



Source: Esser et al., 2020.

Figure 1.3: Type 2 diabetes aetiology models involving primary insulin hypersecretion and β-cell malfunction.

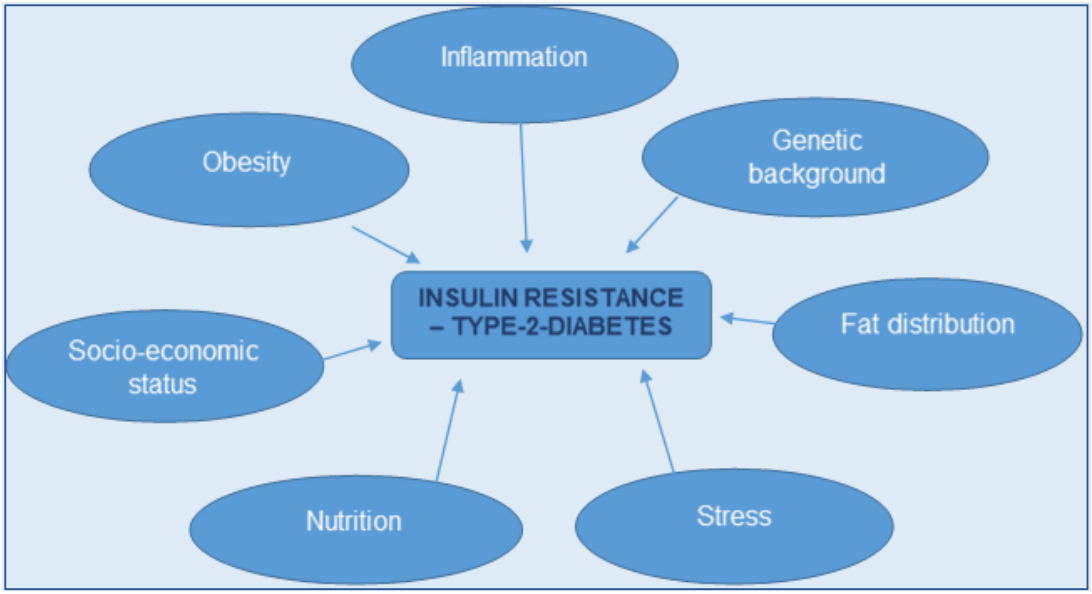
1.3 Disparities in insulin resistance

The incidence and prevalence of T2D differs between black women of African ancestry and white women worldwide (Pham et al., 2019; Hasson et al., 2015). The ethnic disparities appear already in the metabolic characteristics of T2D, specifically

IR (or decreased insulin sensitivity), β-cell (dys)function and HIC (Hasson et al., 2015; Osei and Schuster, 1994), being detected already in childhood (Arslanian et al., 2002; Goran et al, 2002) and making these ethnic groups differently susceptible to diabetes.

Next to the biological differences in glucose metabolism, other factors contribute to the development of IR. These include genetic background, environmental/life-style factors such as obesity, physical inactivity and SES, dietary factors including lack of nutrients or poor quality of dietary composition, and stress (**Figure 1.4**) (Del Prado et al., 2002).

In African-American women, the increased IR is manifested with hyperinsulinaemia caused by the reduced HIC (Fosam et al., 2020 and Osei et al., 1994). The first-pass HIC was two-thirds lower in these women than in the European American counterparts, while extra-hepatic insulin clearance did not differ (Piccinni et al., 2017). In another comparison, the African-American children had lower HIC than Caucasian children, as HIC compensated for their lower insulin sensitivity and secretion (Gower et al., 2002).



Modified from Del Prado et al, 2002.

Figure 1.4: Some of the contributing factors to the multifactorial origin of IR (and thus T2D) next to β-cell dysfunction and reduced HIC.

More recent report of Ishimwe et al. (2021), points on β-cell failure rather than IR as the main etiological determinant of dysglycaemia in African Americans. This phenotype of glucose regulation abnormality is associated with less sever hyperglycaemia and lipid profile and the individuals are not always obese when compared to Africans with glucose intolerance due to IR.

The South African black women were originally thought to be insulinopenic ("lack of insulin secretion") (van der Merwe et al., 1998) but later research indicated that the young normoglycaemic African women have an appropriate β-cell function for the level of insulin sensitivity and compensate for IR by increased insulin response to maintain normoglycaemia. Hyperinsulinaemia in black South African women is then associated with both an increased insulin secretion and reduced HIC (Goedecke[a] et al., 2009; Goedecke et al., 2013). With increasing age, the insulin sensitivity remains unchanged while the insulin secretion declines to the levels seen in their male counterparts (Goedecke et al., 2016).

Even in normoglycaemic individuals, IR can predict future progression to T2D (Taylor, 2012). Therefore, early identification of IR is important as a prompt therapeutic intervention may prevent or delay the diabetes onset.

1.3.1 Obesity and fat distribution in insulin resistant

Most people with T2D are overweight or obese and obesity is a strong component in the aetiology of IR (Gutch et al., 2015). There is a high prevalence of obesity among African-American as well as black South African women (not men). In South Africa, 87% of T2D cases are thought to be caused by excess bodyweight, indicating that obesity is a key contributing factor to the epidemic. Depending on the demographic group, recent studies estimate the age-adjusted prevalence of T2D at either 13.1% or 26.3% (Pheiffer et al., 2021).

However, there is reported higher IR in African-American and black South African women (Goedecke et al., 2013) in comparison with BMI-matched white women, despite more favourable fat distribution (less visceral and more sub-cutaneous fat; less central and more gluteofemoral adiposity) (Resnick et al.,1996; Van der Merwe et al., 2000, 2001; Goedecke[b] et al., 2009) in the former group. In a study of Haffner et al. (1996), non-diabetic African-American women were found to be more insulin resistant than their non-diabetic white counterparts even after adjusting for adiposity. Therefore, the IR irrespective of the degree of adiposity can be the major contributor to the increased prevalence of T2D among African-descent women (Wang et al., 2014). Keswell et al. (2016), reported no differences in IR between South African black and white women, irrespective of differences in the fat distribution. Therefore, adiposity may not be a direct link to higher prevalence of T2D or IR.

In obesity, the liver becomes insulin resistant, which results in an increase in HGP and triggers an inflammatory response (Ying et al., 2018). This is associated with increased levels of pro-inflammatory cytokines and chemokines (see Chapter 1.3.2 below).

1.3.2 Obesity and Inflammation

In obesity, a low-grade chronic inflammation is a key mediator of impaired insulin signalling and development of IR in insulin target metabolic tissues, namely adipose but liver, skeletal muscle and others (de Luca, et al., 2008). The mechanism involves a chronic activation of the immune system (Stępień, et al., 2014). The process is associated with an increased secretion of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukins 6 (IL-6) and 8 (IL-8)) and chemokines (e.g. monocyte chemoattractant protein-1; MCP-1) and adipokine leptin from the excess adipose tissue. They are thought to be the cause of systemic inflammation after entering the bloodstream (Kahn and Flier, 2000). They cause local inflammation in adipocytes. However, the exact mechanisms triggering the inflammatory response in obesity are still not fully understood (de Luca, et al., 2008).

Next to its pro-inflammatory character, leptin controls food intake, energy expenditure and has a role in the regulation of insulin-glucose axis. It contributes to normalisation of hyperglycaemia and hyperinsulinaemia and acts to increase insulin sensitivity. Leptin plasma levels are elevated proportionally to the body fat mass (Amitani et al., 2013).

Another adipose tissue-derived adipokine, adiponectin, has anti-inflammatory effects and can counteract the negative effects of IL-6, MCP-1, leptin and others (Akash et al., 2013). It acts as INS sensitiser, lowers IR (Devaraj et al., 2004), increases fatty acids oxidation and inhibits HGP (Lihn, et al., 2005). It is negatively correlated with measures of adiposity as obesity down regulates adiponectin levels (de Luca, et al., 2008; Lihn et al., 2005). Systemic IR may arise as a result of a leptin and adiponectin imbalance. Adipokines serve as biomarkers for IR due to their ability to reflect the presence of persistent low-grade inflammation in adipose tissues (Sjoholm and Nystrom, 2006).

Pro-inflammatory cytokines are derived mainly from activated macrophages and up-regulate inflammatory reactions. Cytokine IL-6 impairs insulin signalling, lipolysis and endothelial function. IL-6 production is increased by activation of the sympathetic nervous system, e.g. stress (Devaraj et al., 2004). IL-6 is used as an inflammatory biomarker. Chemokines regulate cell trafficking. MCP-1 attracts monocytes from

blood to inflamed tissues. Its signalling is involved in the development of obesity and induction of IR (de Luca, et al., 2008; Panee, 2012).

1.3.3 Socio-economic disparity and nutrition

Well-documented socio-economic disparities in nutrition (Dubois and Girard, 2001) contribute to an explanation of some of the observed social health inequities. High SES individuals are more likely to have healthier eating habits, whereas low SES individuals have dietary profiles that are less consistent with dietary guidelines or nutritional recommendations, which contributes to their worsening health status (James et al., 1997). Therefore, areas of active public health concern include socioeconomic disparity as well as diet quality, as shown by healthy dietary behaviours.

A study of Lindquist et al. (2000) showed that African-American children had significantly higher dietary intake of fruits and vegetables and lower dairy intakes than white children. However, they still had significantly lower insulin sensitivity and higher insulin secretion and greater risk for future development of T2D than white children. This relationship sustained even after adjustment for body composition, SES and dietary patterns.

1.3.4 Genetic background

There are over 400 genetic variants known for their increasing risk of T2D, therefore race-specific variations in these genes could explain the ethnic differences in glycaemic control between African-ancestry black women vs white counterparts (Hasson et al., 2015). However, the contribution of these genes accounts to only about 20% of the total risk. The remaining factors are environment/ lifestyle-based (**Figure 1.4**) (Bergman et al., 2019). Nevertheless, race-specific variations in genes studies showed that HIC can be inherited and it has been suggested that lower HIC in African women may be mainly of a genetic origin supported by central obesity, non-alcoholic fatty liver disease/ metabolic dysfunction-associated steatotic liver disease (NAFLD/ MASLD) and subclinical inflammation (Bergman et al., 2019; Semnani-Azad et al., 2019). Similarly, the relationship between insulin sensitivity and

insulin secretion may depend on the differences in population's genetic and evolutionary history (Kodoma et al., 2013; Brownrigg et al., 2023).

Other gene-environment interactions may be involved. A genetic predisposition for adipose tissue expandability and fat storage capacity has been reported (Yaghootkar et al., 2020). Some genetic variants have safer body fat distribution (storing additional fat in subcutaneous adipose tissue (SAT) vs visceral adipose tissue (VAT) (Yaghootkar et al., 2020)). It is known that some of the deoxyribonucleic acid methylations (in gluteal SAT) were associated with IR and systemic inflammation in black South African women but not in white South African women (Goedecke and Mendham, 2022).

1.4 Assessment of insulin resistance

1.4.1 Measurements of insulin and insulin resistance

Research methods: The most accepted methods for measuring IR are limited to research settings due to their invasiveness and complexity. The clamp studies specifically measure insulin-mediated glucose uptake under controlled conditions. These include the euglycaemic clamp (considered the gold standard) and the hyperinsulinaemic clamp. The insulin sensitivity test and the short insulin tolerance test are less invasive. Less direct methods include assessments of IR based on mathematical models, including the Homeostasis Assessment Model (HOMA) (Matthews et al., 1985; Wallace et al., 2004) and the Quantitative Insulin Sensitivity Check Index (QUICKI), Continuous Infusion of Glucose with Model Assessment (CIGMA) or Frequently Sampled Intravenous Glucose Tolerance Test (FS IVGTT) with minimal model analysis (Bergman et al., 1979).

1.4.2 Diagnosis of abnormal glucose metabolism

WHO has recommended four recent diagnostic tests for diabetes, including measurement of fasting plasma glucose and 2-hour post-prandial plasma glucose

after a 75 g oral glucose tolerance test (OGTT), glycated haemoglobin (HbA1c) and random blood glucose with following criteria: Individuals with fasting plasma glucose values of ≥ 7.0 mmol/L (126 mg/dl) or 2-hour post-prandial plasma glucose ≥ 11.1 mmol/L (200 mg/dl) or both (WHO, 2019); HbA1c of ≥ 6.5% (48 mmol/mol); or a random blood glucose concentration ≥ 11.1 mmol/L (200 mg/dl) are considered to have diabetes in the presence of signs and symptoms (WHO, 2011). Impaired fasting glucose (IFG) is defined as fasting glucose levels (5.6 mmol/L to 7.0 mmol/L) below the required levels to diagnose diabetes but higher than normal reference interval, while IGT is defined as intermediate state between normal glucose homeostasis and diabetes (7.8 mmol/L to 11.0 mmol/L) at 2-hour of the OGTT (ADA, 2022).

1.4.3 Oral Glucose Tolerance Test

The OGTT assesses the body's ability to metabolise glucose or its removal from the bloodstream. It is used to detect disorders in carbohydrate metabolism (diabetes, gestational diabetes, and prediabetes, IR, β-cell function and others). As a diagnostic test, the OGTT serves better than fasting glucose levels only, especially in cases of suspected diabetes where normal fasting glucose results are detected (Bisht et al., 2011). After following a normal carbohydrate diet for three days, the patient presents for the test after 8 - 14h fasting, not having been smoking or using chewing gum the morning before the test. Only sips of water are permitted (ADA, 2022).

The standard OGTT (WHO) evaluates glucose after a standard load of 75 g of glucose dissolved in 200 millilitres of water drunk over a 5-minute period. The samples are collected at fasting and 120 minutes (as per the results interpretation guidelines) from the oral load. Numerous clinical and epidemiological studies use additional sampling points or the duration of the test is extended beyond 2 hours, depending on the purpose of the test. Next to the fasting and 2-hour sampling times, other common sampling points are at 30, 60, (90) or 180 minutes (Eyth et al., 2023).

The next section will introduce chromogranin A (CgA), its structure, intracellular and extracellular functions, factors affecting its levels and further CgA-derived peptides and the shared pathways with glucose regulatory hormones. Specifically, focus will be on the CgA cleavage product CST which showed numerous metabolic beneficial effects in animal-based but human studies and its counterbalancing peptide pancreastatin (PST) in their glucose homeostasis maintenance roles.

1.5 Chromogranin A

Chromogranin A is a glycoprotein of 439 amino acid residuals. It is the most studied member of a family of uniquely acidic granin proteins that includes further chromogranin B and secretogranins II-VIII (D'amico et al., 2014). Granins are found in the secretory granules of many endocrine and neuroendocrine cells, including pancreatic islets (Muntjewerff et al., 2018). They include multiple dibasic sites for intra- and extracellular cleavage to generate tissue-specific biologically active peptides. The secretory granules are essential for the regulated secretory pathway, where pro-hormones (and pro-neuropeptides) are sorted, cleaved during process of the granules maturation and finally secreted into the circulation for their physiological actions (Kim et al., 2001). Originally, CgA was detected in adrenal medulla, where it was co-stored and co-released with catecholamines.

Chromogranin A intracellular function: Within the cells, CgA is a major regulator of the neuroendocrine cells secretory granules' biogenesis and protein sequestration (Kim et al, 2001). Specifically, in pancreatic β-cells, CgA shares the same synthetic pathway with insulin precursor, hormone proinsulin (Wollam et al., 2017), facilitates its transport (next to other peptide hormones) into the immature granules, where it becomes cleaved in the process of their maturation (Wollam et al, 2017). Upon a specific physiological or pharmacological stimulus, the content of the granules (including CgA, CgA cleavage products, insulin, C-peptide and other secretory products) is released by exocytosis into the portal circulation (Loh et al., 2012; Herold et al., 2018). The effect of CgA deficiency in this respect is still not very well understood. A study of Wollam et al. (2017), using a model of CgA deficient (CgA-

KO) mice, demonstrated increased glucose-stimulatory effects on insulin secretion and the secretory pathway in comparison with the wild type control mice. CgA may therefore be an important regulator of optimal secretory function of β-cells.

Other major intracellular role of CgA is in calcium homeostasis and binding; CgA has high binding capacity but low affinity for Ca^{2+}. It accumulates Ca^{2+} within the granules, forming so large intracellular calcium pool (D'amico et al., 2014).

Factors affecting CgA levels. Normal CgA plasma levels are from 0.5 nmol/L -1 nmol/L (Muntjewerff et al., 2018). High plasma CgA concentrations are found in neuroendocrine tumours, where, although non-specific, CgA is considered as a sensitive and still the most valuable universal non-invasive marker in these tumours' detection and treatment monitoring (Gut et al., 2016). To various degrees elevated (2 to 4 times above the cut-off level) concentrations of CgA are associated with some systemic diseases or non-cancerous conditions including renal and hepatic failure, neuroendocrine and inflammatory diseases, some gastro-intestinal disorders and treatment with proton pump inhibitors or H2-receptor antagonists, essential hypertension, hypercortisolism, hyperparathyroidism and hyperthyroidism (Glinicki et al., 2011 Gut et al., 2016). In T2D patients, especially in the poorly controlled individuals, CgA plasma or salivary levels are significantly higher in comparison with healthy controls (Herold et al., 2018).

Chromogranin A is elevated after food ingestion or exercise with the maximum reached at 30 minutes to 90 minutes after a meal and magnitude of about 2 to 3 times the upper reference interval. For this reason, the CgA sampling should be done after rest and fasting (Gut et al., 2016).

Age has only a weak effect on CgA, causing a mild elevation with aging. This might be due to the presence of hypertension in elderly people (Ferraro et al., 2016). CgA is elevated during menopause (probably due to the increased sympathetic tone) and pregnancy (D'amico et al., 2014).

Some authors (Braga et al., 2013) found significantly higher CgA levels in females than males; others found no difference, while Glinicki et al. (2011) reported higher CgA levels in males. Ethnic differences in CgA concentrations remain inconclusive.

The plasma CgA levels increased with significant stressful events showing U-shaped dose response pattern. An optimal amount of CgA might be required for its appropriate function (Herrera et al., 2023).

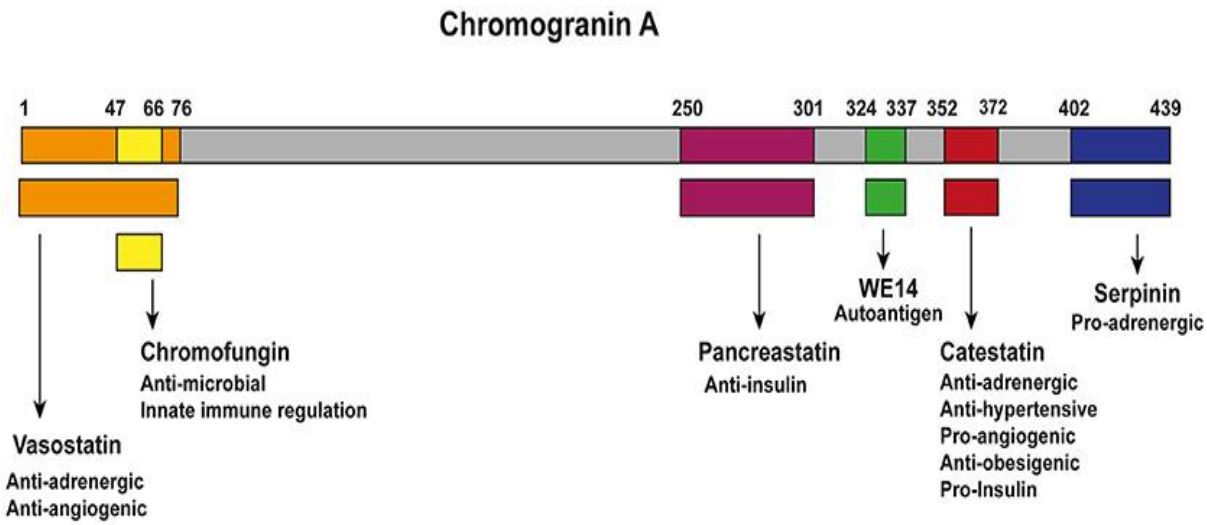
Chromogranin A extracellular function: Outside of the cells, CgA functions mainly through the action of its biologically active and tissue specific cleavage products (Herold et al., 2018). However, often this includes a complex interplay of the CgA system; the intact molecule and the derived proteins, in various other systems regulations (see Chapter 1.5.1 below).

The CgA-knock out (CgA-KO) mice have higher catecholamine concentrations, are hypertensive (Mahapatra et al., 2005), obese on normal chow diet with higher adipokine and lower IL-6 and lipid levels (Gayen et al., 2009), indicating a regulatory role of CgA in blood pressure, obesity and associated inflammation and the cardiovascular health. They have significantly higher glucose stimulated insulin secretion after glucose administration and IR in muscle cells. Lower triglyceride levels have been found in CgA-KO mice, despite similar amounts of fatty tissue (Bandyopadhyay et al, 2017; Mahata and Corti, 2019). Clear endocrine function of CgA has not been identified yet.

1.5.1 Cleavage products of CgA

The CgA accounts for 10 pairs of amino acids as potential cleavage sites (Stridsberg et al., 2004); therefore, CgA has a function of prohormone. About 50 % of CgA is cleaved within the secretory granules, although further extracellular proteolytic processing after its excretion may occur (Herold et al., 2018). CgA processing rate or type is tissue-specific. While e.g. in the adrenal medulla the CgA cleavage rate is

slow, in pancreatic endocrine cells is much faster. The resulting peptides regulate different physiological functions (D'Amico et al., 2014), **Figure 1.5**.



Adapted from: Muntjewerff et al., 2018.

Figure 1.5: Human CgA and its major cleavage products with their functions. Six known major biological active peptides from human CgA cleavage listed from the CgA N-terminus are Vasostatin (VS; hCgA1−76), Chromofungin, (hCgA47−66), Pancreastatin (PST; hCgA250−301), WE-14 (hCgA324−337), Catestatin (CST; hCgA352−372) and Serpinin (hCgA402−439) at the C-terminus.

Any dysregulation in the CgA proteolytic cleavage will disturb homeostasis and will be pathogenic (Ying et al., 2018). This may be in case of CgA hyper-glycosylation. While CgA glycosylation is a part of a post-translational regulatory mechanism, excessive glycosylation impairs the molecule processing into its active fragments which was e.g. associated with increased mortality among acute heart failure patients (Ottesen et al., 2017). Some of the cleavage products have contrary effects in order to balance and maintain the metabolic/circulatory homeostasis (Mahata et al., 2010). In pancreatic endocrine cells, the CgA molecule is cleaved to different cleavage products according to the cell types (Portela–Gomes, Stridsberg, 2001). The fragments with opposing effects, which are involved in the maintenance of glucose homeostasis, are CST (in pancreas secreted by β-cells) (Cetin, 1993) and

pancreastatin (PST; secreted by pancreatic α-cells) (Loh et al., 2012). The latter peptide, PST, is a negative regulator of glucose homeostasis and insulin sensitivity (Muntjewerff et al., 2018). Catestatin is the central focus of this study due to its direct INS-like effects (see below).

1.6 Catestatin

Physiological functions. Pleiotropic hormone CST (hCgA352−372) inhibits catecholamine secretion; it has anti-hypertensive and anti-inflammatory effects, reduces cardiac contractility and is a potent vasodilator (**Figure1.5**) (Mahata et al., 2010). For this project, the most important role of CST is the regulation of glucose and lipid metabolism. Contrary to PST, CST has insulin-sensitizing effects (Muntjewerff et al., 2018). To maintain a balanced glucose homeostasis, both CST and PST level has to be kept at equilibrium (**Figure 1.6**).

Human research studies on CST impact in this field are very sparse. The published studies mainly utilise the CgA-KO, CST-KO and diet induced obesity (DIO) mice models (**Table 1.1**), comparing the baseline with the physiologic effects after CST treatment. The CgA-KO or CST-KO mice are obese on normal chow diet, with hyperinsulinaemia and IR when compared to wild type mice. Supplementation of CST reverses these effects. Catestatin reduced HGP (inhibited gluconeogenesis), induced glycogenesis *(*Bandyopadhyay et al., 2022), improving glucose tolerance and insulin sensitivity (**Table 1.1**).

Catestatin might be involved in the regulation of HIC; however, this action is still unclear; CST-KO insulin-resistant mice had higher insulin concentrations than wild type mice but lower insulin secretion (Borges et al, 2013) (See Table **1.1**). Catestatin supplements reduced insulin circulatory levels and improved insulin sensitivity. However, corresponding cell culture lines treated with CST showed increased insulin secretion, independently of glucose trigger. It has been suggested that CST may increase HIC. On the other hand, in other report, the CST-KO mice were hypertensive and had high HIC and low circulatory insulin. Glucose-stimulated insulin secretion from pancreatic β-cells was not adequate for blood glucose

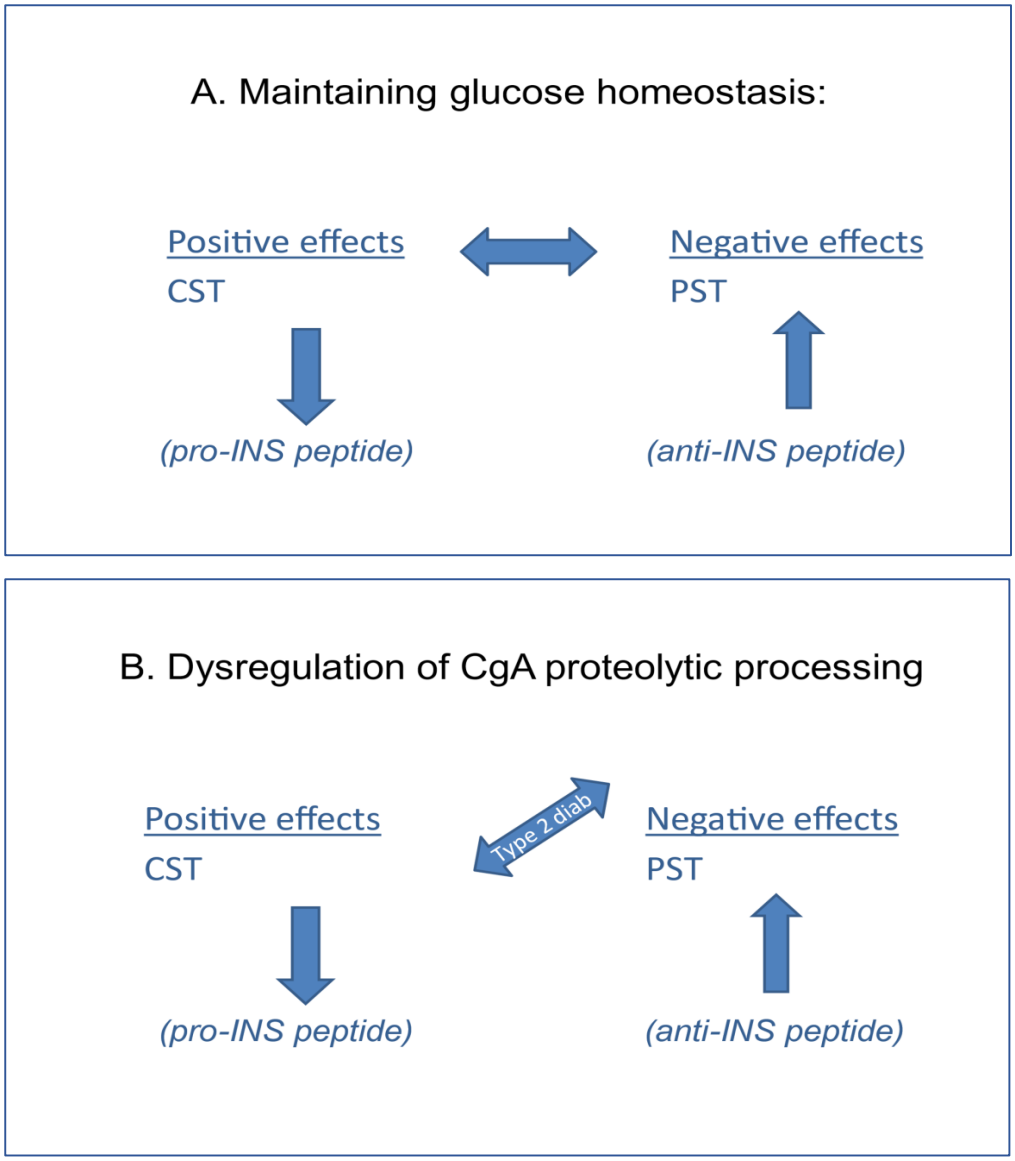
disposal. It was hypothesized that CST may facilitate reduction in HIC to maintain normal glucose tolerance *(https://grantome.com/grant/NIH/I01-BX000323-04)*.

In contrast to insulin, CST has beneficial effects in promoting lipid metabolism. It inhibited lipogenesis and increased lipolysis in adipocytes and fatty acid oxidation and reduced fat in the liver and obesity. The obesity lowering effect of CST is linked to its enhancing action on leptin receptor signalling and to its ability to inhibit α-2 receptor signalling (Bandyopadhyay et al., 2017) (See **Table 1.1**). It has been reported that obese children and adolescents have lower serum CST levels if compared with healthy controls (Herold et al., 2021). In rodents' study, an administration of CST to control lean mice showed no effect on insulin or glucose tolerance (Ying et al., 2018); therefore, CST acts only on obese models. It showed that CST had direct anti-inflammatory effects and reduced cytokines and chemokines, reversing the IR in DIO mice (Ying et al, 2018).

From the above examples of research evidence, although mostly coming from animal or *in-vitro* studies, it is obvious that CgA and CST play significant roles in insulin - glucose and lipid metabolism and that the hyperinsulinaemia seen in IR in the black female population, might be associated with low CST levels, which could be attenuated by obesity. Low CST levels may result from dysregulated CgA cleavage (O'Connor et al., 2009) with increased proportion of PST.

The mechanism of CST actions and the triggers for CgA cleavage are still not well understood. There are genetic variants of CST, some with protective, others with pathological effects (Loh et al., 2012). Twins study demonstrated that CST levels can be heritable (O'Connor et al., 2009).

Due to its numerous beneficial effects, CST was suggested by some authors as a potential novel treatment for hypertension, obesity and T2D (Muntjewerff et al., 2018; Herold et al., 2018).



Drawn by Philisiwe Ntuli.

Figure 1.6: Illustration of maintenance of glucose homeostasis by CST and PST as they have insulin-sensitizing and insulin-opposing effects. **A**: Ballanced effects of CST and PST maintain normal glucose homeostasis. **B**: Dysregulated CgA processing affects glucose homeostasis and leads to pathological conditions.

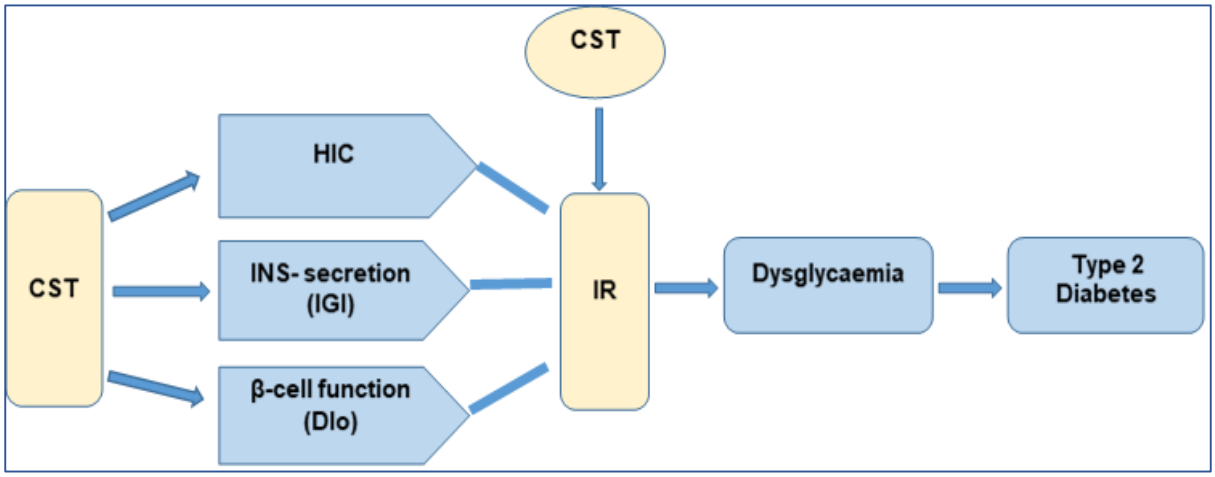
Table 1.1: Regulatory effects of CST in CgA knock-out (CgA-KO) and diet-induced obesity (DIO) mice models. (There is 86% homology between human and mouse CST; Mahata et al., 1997). *GSIS* (glucose stimulated INS secretion)*

	Regulatory Effect	Model	Method/Features	Finding
1.	**Possibly enhances HIC** *(Borges et al., 2013)*	CST-KO mice	Hyperinsulinaemia impaired GSIS*, IR	↓hyperinsulinaemia, improved INS sensitivity but ↑ INS secretion in cell line- ↑ HIC suggested.
2.	**Inhibits gluconeogenesis** *(Loh et al., 2012)*	CgA-KO mice	Hyperinsulinaemic-euglycemic clamp	50% inhibition of hepatic glucose production.
3.	**Inhibits lipogenesis Reduces obesity** *(Bandyopadhyay et al., 2017)*	DIO mice (obese) (High fat diet)		CST treatment – inhibit. expansion of adipose tissue. Promoted fatty acids uptake into liver for oxidation via ↑ leptin rec. signal and ↓alpha-2 adrener.rec. signalling.
4.	**Improves glucose tolerance and INS sensitivity, reduces HGP ;** *(Ying et al., 2018)*	Syst. CST-KO mice	Gains weight on normal diet; has ↑HGP, ↑Glucose and ↑ insulin	CST treatment – normalised HGP, Glucose and INS (Decreased expression of gluconeogenic genes).
5.	**Reduces liver fat, obesity Anti-inflammatory function in reduction of IR;** *(Ying et al., 2018)*	CST-KO, DIO mice (High fat diet → → obese)	Increased CgA/CST Low CgA, Low CST, excess liver fat, inflammation	CST treatment – reduced hepatic/plasma lipids Reduced plasma INS; improved glucose tolerance Reduced cytokines and chemokines ↓ expression of pro-inflammatory genes, ↑ Expression of anti-inflammatory genes No effect on control wild type mice, unless obese.

HIC; hepatic insulin clearance, CgA-KO; chromogranin A knock out, C-PEP; C- peptide, INS; insulin, CST-KO; catestatin knock-out, DIO; diet-induced obesity, HGP; hepatic glucose production

1.7 Aim of the study

The aim of the study is to determine whether circulatory levels of CST (or CgA; CgA/CST ratio) differ between healthy normoglycaemic black and white South African women independently of obesity and whether they are significantly involved in the regulation of IR (directly or through other mechanisms involving factors such as HIC, insulin secretion or β-cells function; See Chapter 1.1.4). Is the CST regulatory effect different between black and white women?



CST; catestatin, IGI; insulinogenic index, DIo; oral disposition index, IR; insulin resistance

Drawn by Philisiwe Ntuli.

Figure 1.7: Diagram of the study research focus: Investigation of the CST (CgA or CgA/CST) direct effects on IR or on the IR-associated factors such as HIC, INS secretion or β-cell function.

1.8 Study Objectives

1. To describe and compare the basic characteristics of the study population (anthropometry, demographic and socio-economic data, metabolic variables from the OGTT-collected samples and derived indices).
2. To determine the basal insulin and IR levels in both ethnic groups and to identify, whether there are significant differences between them as anticipated from the literature.
3. To determine whether CST levels are significantly lower (CgA/CST ratio higher) in the black African women vs. white women, as we hypothesize. Further, evaluate differences in CgA.
4. To identify whether there are any significant associations of CST (CgA or the CgA/CST ratio) with IR or the indices of insulin dynamics (based on the variables derived from the OGTT) and the confounders (anthropometry, age, markers of adiposity and inflammation, demographic and SES variables).
5. To determine whether CST (CgA or the ratio) affects significantly insulin, IR and the indices of insulin dynamics, when corrected for age, BMI (or any other strongly associated anthropometry index) and confounders (obesity and inflammation markers, family income and absence/presence of family genetic disease).

Chapter 2

2. Methods - Study population and sampling

This clinical laboratory prospective quantitative study took place in the province of Gauteng, South Africa between February 2021 and October 2023.

2.1 Study Participants

The participants were recruited from the female staff members at the National Health Laboratory Services (NHLS) at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), the staff and students of the University of the Witwatersrand (Wits) in Parktown and further from the NHLS-Braamfontein branch, the Wits Donald Gordon Medical Centre in Johannesburg and the Pathcare Vermaak Laboratories in Centurion, Gauteng province. The aim was to enrol one hundred and twenty "apparently healthy" local black and white women, sixty for each population group. The nationality was verified from the participant's South African identity document.

2.2 Enrolment and study design

2.2.1 Inclusion criteria

- Black (of South African ancestry) and white (long term residents) South African women with age group between 18 to 50 years and BMI>=18 to <= 46 kg/m^2.
- Non-diabetic with no known chronic or acute disease (such as cardiovascular disease, chronic heart failure; high cholesterol or atherosclerosis; kidney dysfunction; cancer, anaemia or any other blood disorder; inflammatory disease; thyroid disorder; chronic obstructive pulmonary disease (COPD); HIV; TB- treated or untreated.
- No current treatment with proton pump inhibitors, antihistamines or steroid hormones or anti-coagulants (which could influence CgA and/or CST levels).
- Non pregnant, not lactating or being (post)menopausal.

- Normoglycaemic (normal fasting and 2-hours' glucose) see Chapters 1.4.3 and 2.4.3, (WHO criteria, 2019).
- Normotensive (BP<140/90 mmHg) (WHO criteria, 2019) without treatment

2.2.2 Exclusion criteria

- Women of other ethnicity than South African Black and White.
- Below 18 years or above 50 years of age.
- Women with BMI <18 and >46 kg/m^2
- Menopausal (women on hormonal adjustment therapy) and post-menopausal women.
- Women who have been diagnosed or who were currently treated for any of the following conditions or diseases as these conditions are known to affect the CgA levels:
    - Diabetes or abnormal blood glucose test
    - Cardiovascular disease; chronic heart failure
    - High cholesterol; atherosclerosis
    - Hypertension
    - Kidney disease/dysfunction
    - Any cancer, anaemia or any other blood disorder
    - Current treatment with anticoagulants
    - Inflammatory diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus (SLE), inflammatory bowel disease)
    - Current treatment for heartburn/ulcers (Omeprazole/ Trustan/Nexium etc.)
    - Current treatment for allergies (antihistamines)
    - Current treatment with steroid hormone (cortisone)
    - HIV or TB
    - Thyroid disorder; chronic obstructive pulmonary disease (COPD)

2.3 Enrolment methods and study procedures

The enrolment methods included Interview (enrolment questionnaire), non-invasive methods like blood pressure (BP) measurement to verify normotensive status and lastly, invasive measurements (blood collections, see Chapter 2.4) - for the verification of the normoglycaemic status.

The actual study included anthropometrical data collection, Interview (SES questionnaire), and blood collection during the OGTT.

For the convenience of the participants and to simplify the study proceedings, some parts of the enrolment and of the study procedure were conducted at the same appointment.

2.3.1 Enrolment Interview

The participants were recruited by advertisements placed on the boards, lift-waiting and other public areas in the enrolment facilities (Invitation Letter-**Appendix E**/poster, **Appendix I**) or approached directly or by e-mail. Interested women were either invited to the Department of Chemical Pathology, NHLS at the Wits Medical School for an interview or they were attended to and interviewed at the place of the enrolment in case of the external (other than the Wits Medical School and CMJAH) enrolment points. The study was explained to them and volunteers were evaluated for the compliance with the study enrolment criteria using a Study Inclusion Questionnaire (**Appendix F).** This included questions on alcohol consumption and smoking habits.

All volunteers who fulfilled the pre-selection criteria and signed the Study Informed Consent (**Appendix H**) were enrolled at this stage. For better clarity and understanding of the sequence of procedures, a simple study flow diagram was included in the Informed Consent. In the next step, the participants were invited for anthropometric measurements, BP verification and blood samples collection after overnight fasting.

2.3.2 Anthropometric data and blood pressure measurements

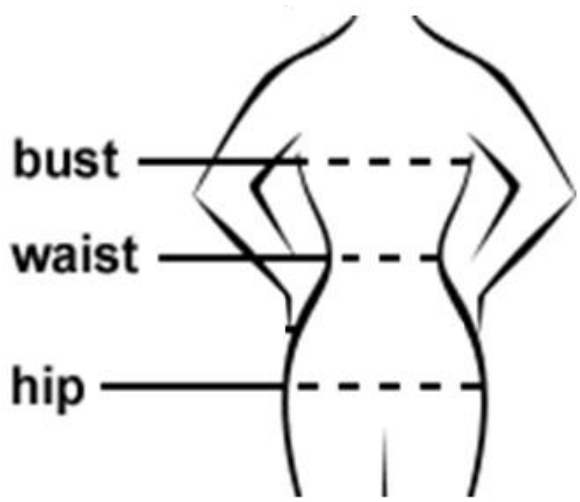
The participant's height (wall mounted stadiometer BioCare; BioClin Solutions, South Africa), weight (electronic body weight scale, Casa (Pty) Ltd, South Africa), waist (at the level of umbilicus) and hip (largest gluteal area; see Figure 2.1) circumferences were measured using an ordinary non-elastic tape measure after removal of any heavy clothing/shoes (measurements taken to the nearest 0.1 kg and 1cm). Body Mass Index was calculated according to the formula: BMI = weight [kg] ÷ height^2 [m^2] (Keswellet al., 2016). This index is frequently used body measure for an assessment of general and central obesity (Kuang et al., 2022), although it does not distinguish between adipose and muscle tissue. Other adiposity measures and indices were collected and calculated.

Waist circumference (WC) and hip circumference (HC) were used to express their ratio; waist/hip ratio (WHR). Waist circumference is a marker of abdominal adiposity and for a given BMI is considered as an acceptable proxy measure of visceral adiposity (Romaguera et al., 2010), although cannot differentiate between contributions of abdominal visceral adipose tissue (VAT) and abdominal subcutaneous adipose tissue (SAT) to central obesity. Waist circumference is associated with the increased risk for chronic diseases such as T2D, cardiovascular diseases and some others. Hip circumference is a proxy measure of the "protective" gluteofemoral fat (Manolopoulos et al., 2010). Waist/hip ratio reflects then a fat distribution and evaluates central adiposity (McLaughlin et al., 2011).

Waist to height ratio (WHtR) is another proxy measure of visceral adipose tissue (Savva et al., 2000) and it was found as a more suitable anthropometric index for IR screening than BMI or WC (Anoop et al., 2021). And finally, Body Shape Index (ABSI), independent of BMI, was calculated as follows: ABSI =WC/BMI$^{2/3}$ ×height$^{1/2}$ (Bertoli et al., 2017).

Blood pressure was evaluated in sitting position after resting; using automatic upper arm digital BP meter (Electronic Sphygmomanometer, Wise Medical Care for Health,

China) and the average of two readings was recorded. A standard adult cuff size was used for all the participants.



https://www.vaikobi.com/pages/size-chart-women

Figure 2.1: indicating the points of measurements for hip and waist circumference measurements

All the above measurements were taken in the Chemical Pathology department of the NHLS for participants from the Wits Medical School and CMJAH-NHLS. Measurements for participants from external areas were taken at the particular enrolment points. All normotensive women matching the study enrolment BMI criteria advanced to the next step of the blood samples collection and filled in a questionnaire on the SES.

2.3.3 Socio-economic status

Information on SES was collected from participants in a form of a self-administered questionnaire (**Appendix G**) (Toman, 2011). This included participant's level of education (ranging from illiterate to tertiary education in 5 categories), welfare assessment in a form of a family monthly income (ranging from below R 10 000 to over R60 000 in 5 categories) and the number of adults (>=18 years old) or children (<18 years old) in the household. Since only students or employees were targeted for the study, the employment status was taken away.

2.3.4 Sample collection

The main study step included blood sample collection. Participants from the Wits Medical School and the NHLS at the CMJAH were then booked for an OGTT. The participants from the other enrolment points were booked for only fasting blood sample collection (See section 2.5).

2.4 Oral glucose tolerance test

Participants were instructed to come between 7:30 am - 8:00 am after overnight fasting (i.e. no food or drink from 10.00 pm night before) to the departmental Day ward in the CMJAH, where the 75 g-OGTT (WHO Criteria, 2006) was carried out with the help of the Day ward-employed nurses. Since the ethnic disparities were reported to be associated mainly with early insulin secretion (Hasson et al., 2015; Gower et al., 2002), the OGTT sample collection was reduced to the first 30 minutes, to enable calculations of the IGI and DIo. The nurse in the Day Ward collected a venous blood sample from the sitting participant at fasting (before glucose drink) and at 30 minutes after drinking a glucose drink over 5-minute period. The glucose drink contained 75 g of dextrose monohydrate (pre-packed, MedicoLab cc, Amalgam SA) dissolved in 200 mL of water.

2.4.1 Blood glucose by glucometer

Test principle: The glucose in the blood sample is converted to gluconolactone by the enzyme on the test strip mutant variation of quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus,* recombinant in *E. coli*. The meter interprets the direct electrical current formed by the reaction into blood glucose results (Roche Diagnostics, 2013).

2.4.2 Glucose evaluation by a finger-prick

To evaluate the glycaemic status at 2 hours, the participant was told to abstain from food and drinks until sampling by a finger prick and an electronic glucose-meter

(Accu-check Performa; Roche) at 2 hours of the OGTT was completed. These values served for the selection of normoglycaemic participants only and were not used in the data analysis. Women with impaired glycaemia (evaluated with OGTT or abnormal fasting glucose levels) were removed from the study, nevertheless, they received their fasting glucose and insulin results.

2.4.3. Evaluation of normoglycaemia (criteria for impaired fasting glucose)

To evaluate IFG, the fasting plasma glucose levels were analysed followed by 2-hour glucose after OGTT analysis to evaluate IGT and to confirm normoglycaemia. Glucose levels falling outside the normal acceptable range for both fasting (< 5.6 mmol/L) and 2-hour OGTT (<7.8 mmol/L) were indicative of IFG or IGT (WHO, 2019).

2.5 External participants' enrolment and sampling

Due to logistic problems, the blood sampling of external participants was performed at fasting only and overlapped with their enrolment. A specific time slot for sampling of multiple participants was negotiated with the external blood collection point so the local phlebotomist could assist with the blood collections. Women were approached beforehand and had the study explained. Volunteers attended the appointment already at fasting, they were evaluated as above (see 2.3.1-2.3.3) and those who complied with the selection criteria had their fasting blood collected.

2.6 Samples handling and storage and measured analytes.

Analytes in the study: Samples for fasting plasma glucose were collected into sodium fluoride tubes (Becton Dickinson (BD) (BD Diagnostics; Franklin Lakes, New Jersey, USA)). Fasting serum (BDSST tubes) was used for the analysis of insulin, C-peptide, CgA, CST and IL-6 while the fasting plasma (BD-EDTA Vacutainer blood collection tubes) was used for the analysis of MCP-1, leptin and adiponectin.

Plasma samples (OGTT-collected) were used for analysis of 30-minute glucose (BD-sodium fluoride tubes) and for 30-minute serum insulin (BD-SST tubes).

Samples handling and storage: Samples were kept in a cooler box and within 30 minutes after the completed collection, samples were spun down using Beckman coulter, Allegra X-22R centrifuge at 3500 revolutions per minute (rpm) for 10 minutes. Obtained serum/plasma was aliquoted and stored at -80°C before the analysis. Set of samples was stored for further research (see Informed consent, **Appendix H**).

Except for the venous blood sampling, all the study procedures and sample analyses were carried out by the researcher.

2.7 Biochemical processing of samples.

2.7.1 Automated systems

Plasma glucose samples were analysed immediately after the separation in Roche Cobas 8000 modular analyser (enzymatic reference with hexokinase, UV detection; Roche Diagnostics, USA). Serum insulin and C-peptide samples were analysed immediately after the separation using Cobas 601 modular analyzer (a “sandwich” electro-chemiluminescent immunoassay; Roche Diagnostics, USA). The researcher performed these analyses; both methods were well established in the departmental routine laboratory.

Processing of samples on the Automated Roche system: First**,** the instrument maintenance, calibration and processing of quality controls were performed. If the calibrators and controls performed according to the instrument’s and the analyte’s package manual (following the manual and laboratory SOP), the patient sample analysis was carried out.

C-peptide kit specifications (Elecsys C-peptide, Cobas- 03184897):

The assay measuring range for serum and plasma was 0.003-13.3 nmol/L with analytical sensitivity limit of detection (LOD) of 0.003 nmol/L. The precision for intra-assay variation was CV ≤ 5.0% and for inter-assay variation CV ≤ 3.8% as reported by the kit manufacturer. Expected reference interval was 0.37-1.47 nmol/L for fasting samples. The unit conversion factors: ng/mL(µg/L) x 0.33 nmol/L.

Insulin kit specifications (Elecsys Insulin, Cobas- 12017547122):

The assay measuring range was 0.2-1000 µU/mL (1.39-6945 pmol/L) with analytical sensitivity of 0.2 µU/mL (1.39 pmol/L). The precision was CV ≤ 4.9% for intra-assay and CV ≤ 3.6% for inter-assay variation as reported by the kit manufacturer. Expected reference interval was 2.6-24.9 µU/mL (17.8-173 pmol/L) for fasting samples.

Principle of the analysis: Enzymatic reference method with hexokinase; Hexokinase catalyses the glucose phosphorylation to glucose-6-phosphate (G-6-P) by adenosine triphosphate (ATP) (Tietz, 2006).

Glucose + ATP ➔ G-6-P + ADP

G-6-P dehydrogenase oxidizes G-6-P in the presence of nicotinamide adenine dinucleotide phosphate (NADP+) to gluconate-6-phosphate. The rate of dihydronicotinamide-adenine dinucleotide phosphate (NADPH) formation during the reaction is directly proportional to the glucose concentration (measured photometrically) (Tietz et al., 2006).

Remaining tests employed commercial manual Enzyme-Linked Immunosorbent Assay (ELISA) kits on 96-well microplate.

2.7.2 Enzyme-Linked Immunosorbent Assays:

Catestatin sample analysis employed a competitive enzymatic immunoassay (EIA) kit.

Serum CgA, leptin, IL-6, MCP-1 and adiponectin kits were analysed using ELISA assay based on a sandwich principle.

All above assays, except for CgA, employed biotin-streptavidin binding, horseradish peroxidase (HRP) as an enzyme in the label and 3,3,5,5'-tetramethylbenzidine (TMB) as a substrate for HRP-catalysed enzymatic reaction. There is a high binding affinity between streptavidin and biotin and the combination of biotinylated antibody and streptavidin-conjugated horse radish peroxide (streptavidin-HRP) utilised in ELISAs enables improved sensitivity of the detection (Lakshmipriya et al., 2016).

The manufacturer's kit inserts were followed in all steps of the assay's preparation, performance and output detection.

2.7.3 Instruments involved in the ELISA assays of the current project:

- The orbital plate shaker and incubator (Thermo star, BMG Labtech, Germany) was used for incubation of the assay microplates.
- The automated plate washer (Biotek, Analytical & Diagnostic Products, USA) was utilised for automated microplates washing procedure.
- The Biotek Synergy HT micro plate reader (Analytical and Diagnostics Products, USA), equipped with Synergy 5.1 software was used as a detection system.
- In further use were vortex (Fine vortex, Sep. Sci, SA) and a centrifuge (Allegra-X-22, Beckman Coulter, CZ). All instruments were available in the Chemical Pathology department.

2.7.4 Principle of utilised ELISA assays:

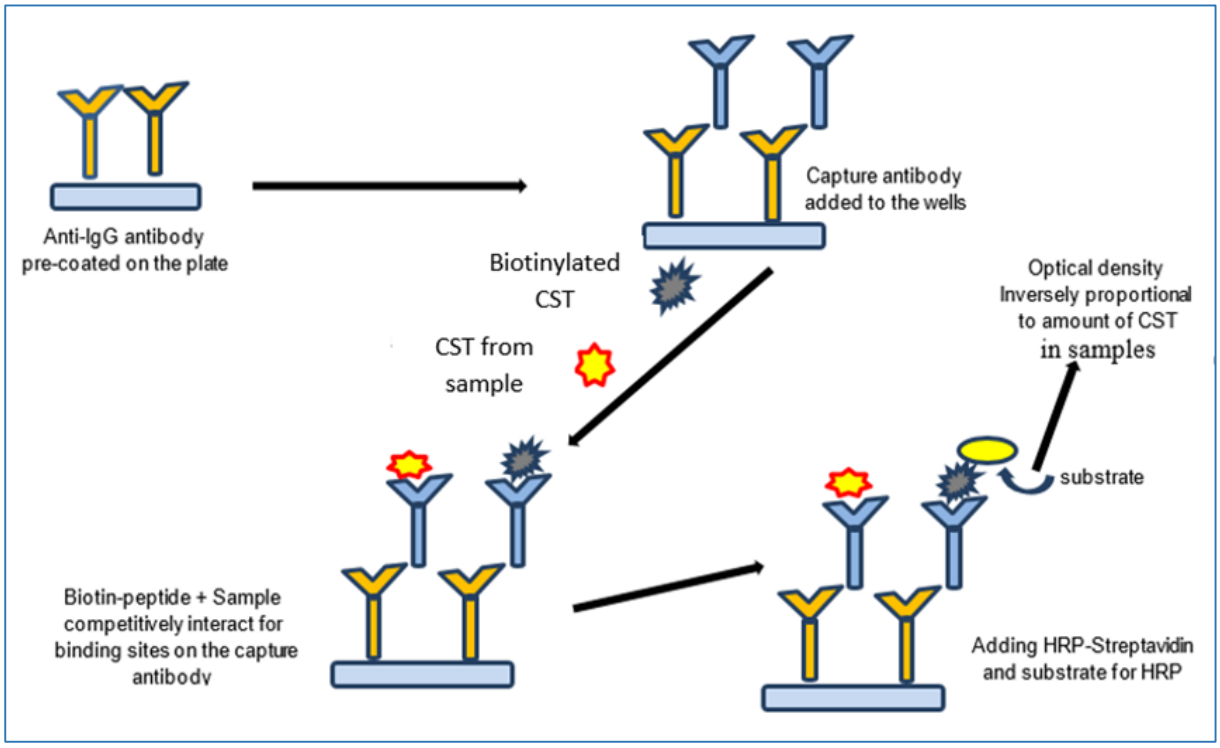
2.7.4.1 Competitive assay:

Catestatin assay (EIA-CAT; Ray Biotech, Norcross, GA, USA)

The CST assay was based on a competitive principle of ELISA in which antigen from a sample and labelled antigen compete for a limited number of binding sites on an antigen (CST)-specific capture antibody. The manufacturer's kit insert was followed.

During the initial incubation step, this antibody was attached to the anti-IgG antibody pre-coated on the walls of a microplate (therefore, binding between two antibodies was utilised in this assay; **Figure 2.2**). Any unbound material was washed off in a subsequent washing step. Sample with CST and biotinylated CST were added into wells to compete for a binding. Following a washing step, a streptavidin-HRP conjugate was added into the wells to react with biotin label on the competing CST. After the last washing step, a substrate (TMB) for the enzymatic reaction catalysed by HRP was added, resulting in a blue colouring of the product, enabling thus a colorimetric detection. The measured signal was indirectly proportional to the concentration of the analyte.

The assay range was 0.1-1000 ng/mL, analytical sensitivity 0.5 ng/mL and precision of CV<10% for intra-assay and CV<15% for inter-assay variation as reported by the kit manufacturer. The assay is designed to detect human, mouse and rat CST; human samples used dilution factor 12x.



Adapted from the Ray Bio CST kit insert.

Figure 2.2: Principle of the CST competitive assay.

This approach utilises binding between two antibodies, where anti-CST polyclonal antibody is captured via binding on the anti-IgG pre-coated on the wells. Analyte (CST from the sample) and CST labelled with biotin are added to the plate to compete for the binding on the capture antibody. Biotin (attached to competing CST)– streptavidin (attached to HRP) binding is utilised to improve detection sensitivity. The addition of a substrate for HRP results in a colour change of the wells content, enabling thus a spectrophotometric detection on a 96-well plate reader.

2.7.4.2 Sandwich (non-competitive) ELISA assay principle (in the detection of sample antigen) is based on two antibodies specific for different epitopes of the measured antigen.

The first antibody is coated on the surface of the 96 well-plate and it is used as a capture antibody to facilitate the immobilisation of the antigen (**Figure 2.3**). After addition of a sample solution into the wells, an incubation step follows to reach the reaction equilibrium. Any unbound material is then removed in a washing step. The second, enzyme-conjugated antibody (known as detection antibody) is added, and the antigen becomes “sandwiched” between these two antibodies (Alhaj et al., 2022) during further incubation. Another washing step follows to remove an unbound fraction. Finally, a substrate addition triggers an enzymatic reaction (catalysed by the enzyme in the conjugate) which leads to a coloured product. The reaction is stopped by a stop solution (acid) to stabilise the final product. This may lead to a further colour change. The resulting colour is measured on a 96-well plate reader (spectrophotometer) at a particular recommended wavelength. The obtained signal (absorbance or optical density) is directly proportional to the sample concentration.

The biotin-streptavidin binding can be employed in a sandwich assay, where the detection antibody is labelled with biotin and HRP is conjugated with streptavidin.

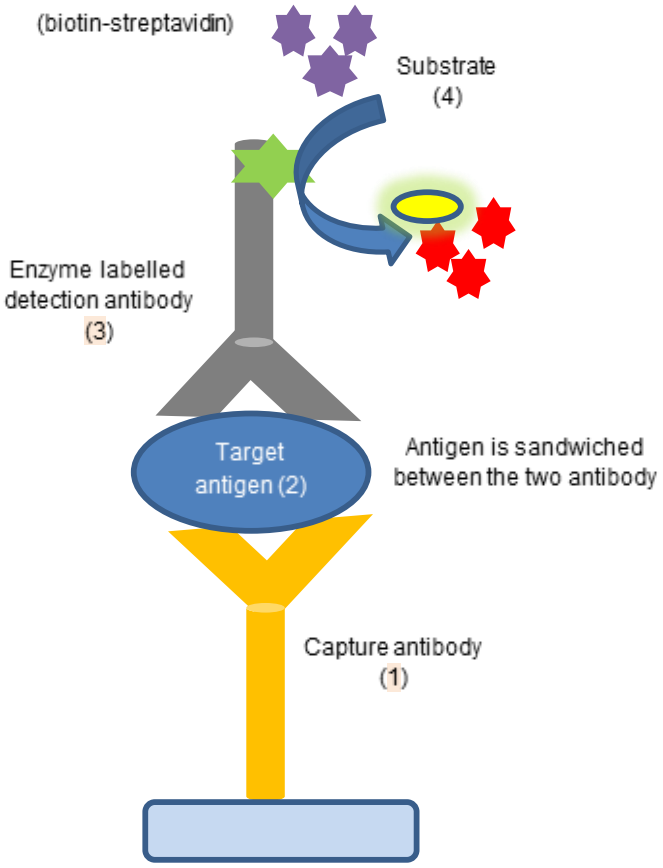
2.7.4.3 Individual sandwich ELISA assay kits with performance details:

- **Chromogranin A** (Ref. C24916; Beckman Coulter, CZ)

Assay kit details:

The assay range was 1.4-900 µg/L, analytical sensitivity of 1.4µg/L, limit of quantification (LOQ) of 2.3 µg/L and with precision of CV ≤ 6.3% for intra-assay and CV ≤ 5.3% for inter-assay variation, as reported by the kit manufacturer. Samples were diluted 21-fold.

Assay specificity. Cross-reactivity: Patients who have been regularly exposed to animals or taking medications like proton pump inhibitors or histamine type-2 receptor antagonists can have serum CgA level affected.



(*Adapted from the ElabScience Leptin kit manual).*

Figure 2.3: Principle of the sandwich assay.

The capture antibody is immobilised, analyte and the detection antibody are added (simultaneously or sequentially) to the wells. Analyte is sandwiched between both antibodies, where each antibody is targeting different binding places (epitopes) on the antigen to omit competition. Enzymatically labelled detection antibody facilitates the enzymatic redox-reaction when substrate is added to the reaction mixture. Resulting colour change is detected spectrophotometrically.

Table 2.1: Individual assay kit details (IL-6, MCP-1, adiponectin, leptin)

	IL-6 (pg/mL)	**MCP-1** (pg/mL)	**Adiponectin** (ng/mL)	**Leptin** (pg/mL)
Assay range:	1.56-100	62.50 - 4000	0.16 -10	15.63 – 1000
Analytical sensitivity: LOD	0.94	37.50	0.10	9.38
Assay specificity: Cross-reactivity	Kit recognizes Human IL-6 in samples. No significant cross-reactivity or interference between Human IL-6 and analogues was observed.	Kit recognizes Human MCP-1 in samples. No significant cross-reactivity or interference between Human MCP-1 and analogues was observed.	Kit recognizes Human Adipon. /Acrp30 in samples. No significant cross-reactivity or interference between Human Adipon. and analogues was observed.	Kit recognizes Human LEP in samples. No significant cross-reactivity or interference between Human LEP and analogues was observed
Assay precision: Intra-assay CV%	<6%	<6%	<7%	<6%
Assay precision: Inter-assay CV%	<7%	<6%	<5%	<6%
Dilution factor	No dilution	No dilution	1000X	20-100x*

*****Exact dilution factors used in these assays were as follows:50X for BMI<35& 100X for BMI>35 (based on the kit insert). *IL-6; interleukin-6, MCP-1; monocyte chemoattractant protein-1, CV; coefficient of variation, LOD; limit of detection, LEP; leptin, Adipon.; adiponectin.*

- **Interleukin 6 (IL-6;** E-EL-H0102; E-EL-H6156**),**
- **Adiponectin** (E-EL-H6122)**,**
- **Leptin** (E-EL-H6017)
- **Monocyte Chemotactic Protein 1 (MCP-1;** E-EL-H6005**)**

All these kits (ElabScience R&D ELISA kits; ElabScience, Texas, USA) employed the same sandwich assay procedure and utilised biotin-streptavidin binding. Further characteristics are displayed in Table 2.1 above.

2.8 Surrogate indices and calculations

Basal glucose, insulin (often used as a measure of IR in clinical setting) and C-peptide (a measure of insulin secretion not affected by HIC) were used to evaluate differences in glucose-insulin-C-peptide dynamics between groups. However, to investigate the effects of CST (CgA or CgA/CST) on glucose regulation, surrogate indices for evaluation of IR, HIC, β-cell function and insulin secretion were calculated based on the data collected at fasting or in combination with 30-minute time points of the OGTT.

2.8.1 Evaluation of insulin resistance/ insulin sensitivity, insulin secretion and hepatic insulin clearance and β-cell function:

The gold standard for the assessment of insulin sensitivity is euglycaemic-hyperinsulinaemic clamp and for insulin secretion hyperglycaemic clamp method (DeFronzo et al., 1979) (See Chapter 1.5.4). However, various surrogate indices have been developed, validated and applied in many epidemiological and clinical studies in the past (Singh et al., 2010) as a cheaper and simplified approach. Although there are numerous surrogate indices in use, for the current study, indices based purely on the data collected from the OGTT were selected. These included:

- **HOMA-IR index to evaluate hepatic IR**: HOMA-IR = (GLC-F [mM] * INS-F [uIU/ml])/ 22.5 (Matthews et al., 1985), utilising basal plasma glucose and insulin concentrations. Estimates of IR derived from HOMA-IR were in a good agreement with the other static or dynamic well-validated methods (Wallace et al., 2004). GLC-F= fasting glucose, INS-F= fasting insulin.
- **Insulinogenic index** (**IGI)** was used as a surrogate measure of early insulin secretion in response to glucose, utilising fasting and 30-minute values of

glucose and insulin from the OGTT; IGI = (INS- 30 – INS-F)/(GLC-30 - GLC-F); It has been used previously (Utzschneider et al., 2006) and validated (Tura et al., 2005); GLC-F=fasting, GLC-30 = glucose at 30 minutes; INS-F = fasting insulin; INS-30 = insulin at 30 minutes..

- **Insulin sensitivity index** (ISI) derived from the basal levels of insulin and glucose was used to evaluate hepatic insulin sensitivity**.** It has been expressed as inverse value of HOMA-IR. ISI = 1/HOMA-IR (Retnakaran, et al., 2011; Utzschneider, et al., 2006).
- **Basal hepatic insulin clearance** (**HIC)** index: C-PEP-F/INS-F. This is a molar ratio calculated from basal levels of C-peptide and insulin and evaluates insulin extraction at steady state (Retnakaran, et al., 2011). This index has been validated previously (Semnani-Azad et al., 2019).
- **Oral disposition index (DIo):** represents a measure of β-cell function derived from OGTT. DIo = (insulin secretion * insulin sensitivity) = (INS- 30 – INS-F)/ (GLC-30 – GLC-F) *1/INS-F . (GLC [mM]; INS-F [pM]; Goedecke, et al., 2009).

2.9 Study confounders

A confounder is defined as a variable that affects the variables of interest in the study in a way that the results do not show a true relationship (Pourhoseigholi, et al., 2012).

Each regression model as described below has been corrected for possible study confounders, which included age, BMI (or the most strongly associated anthropometric measure in the correlation analysis), inflammatory markers IL-6 and MCP-1 and adiposity markers leptin and adiponectin. Further, lifestyle habits (alcohol consumption, smoking) health (family genetic disease) and SES based on a family monthly income, number of adults and children in the household (as measure of overcrowding). Each regression analysis that involved both ethnic groups, included correction for age and BMI due to significant differences in these variables between both ethnic groups and additional adjustment for further selected variables.

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2.10 Sample size estimation

The sample size estimation serves to evaluate the number of samples needed for the statistical data analysis for having a sufficient statistical power to detect existing differences.

a) Studies reporting human CST plasma or serum levels are quite rare. Based on a cardiology study comparing severely ill patients with controls (Xu et al., 2016) using CST EIA kit (Phoenix, USA), the number of participants in each group were estimated from the formula for comparison of two means: $n = ((u+v)^2 * (\sigma1^2 + \sigma2^2))/(\mu1-\mu2)^2$; (Kirkwood, 1998), where u=1.28, v=1.96 (table values at significance levels of 5% and power 90%); $\sigma1$ and $\sigma2$ are standard deviations of each group in comparison and the denominator represents a difference between the groups means ($\mu1$ and $\mu2$). Since the means difference may be smaller in the planned study (no severely ill patients), reducing the above study denominator 0.75 to one half and increasing σ to 1 (from average 0.93) for both groups, 60 women should be enrolled for each group.
b) Based on a regression analysis, correcting for 5-6 confounders at a time, 60 participants in each group were required (Petrie, 2000).
c) Numbers calculated from other variables (glucose, insulin and CgA) support this assessment.

2.11 Statistical analysis

Statistica version 14 (StatSoft, Tulsa, OK, USA) was used for data analysis, the significance level was set at 5% and p values <0.05 were considered significant.

2.11.1 Descriptive statistics

Objective 1: The basic participants' characteristics were presented in a form of a mean with standard deviation (SD) if continuous variable was normally distributed or as a median (lower quartile; upper quartile) for skewed data and summarised in tables. Normality of the data distribution was tested by the Shapiro-Wilk test.

Demographic data that included behavioural factors such as smoking and alcohol consumption (Enrolment Questionnaire, **Appendix F**), life-style factors such as body composition, SES (**Appendix G**) such as family monthly income and health factors such as family genetic background (categorical data) were expressed as frequencies (%) and presented graphically as obtained from the questioners.

Differences in categorical variables between groups were determined by the Chi-squared (Chi^2) test and due to very low numbers in certain categories, these were pulled together and re-coded (for both populations). This is, the minority of participants who were drinking alcohol more frequently were merged with the participants who were drinking alcohol occasionally vs. non-drinkers. Further, five income categories were reduced to three categories of relatively low (originally categories 1&2), medium (3) and high (4&5) income for comparison. Number of adults in a household was re-coded for 1 or 2 vs. 3 & more and, similarly, the number of children as 0&1 vs 2& more. All 2x2 contingency tables were computed by Statistica software. Family monthly income 2x3 contingency table was computed manually and compared with the table values (Kirkwood, 1997, Table A3, Pg. 209). Variable family genetic disease included low frequencies (6) in the contingency table; therefore, the Fisher exact test was applied.

Age, anthropometric variables and their indices, metabolic variables and the surrogate indices were compared using the parametrical t-test for independent groups (see Chapter 2.11.2).

Due to significant differences in BMI and age which were observed between groups, additional analysis adjusting for these two variables has been performed by multiple linear regression. Both unadjusted and adjusted results are presented.

Hyperinsulinaemia, was assessed by comparison of fasting insulin levels against the upper limit of the reference interval for fasting insulin (24.6 mIU/L) used by the NHLS for the particular assay for a diagnostic purpose. High fasting levels of insulin in normoglycaemic individuals reflected IR (Singh et al, 2010).

2.11.2 Analytical statistics

Skewed data was log-transformed prior to analysis (all variables were skewed excluding fasting glucose, CgA, diastolic BP and ethnicity that were normally distributed), except for variables IL-6 and MCP-1 that were normalised by square root transformation. Unpaired Student's t-test was used to compare between both populations groups **(Objective 1)** and derived p-values were entered into the tables (**Tables 3.1 and 3.3** in Results). The adjustment for BMI and age was carried out using multiple linear regression analysis with the variable of interest as the dependent variable and age, BMI and ethnic were entered as predictors.

Body mass index-split tables: In addition to the participants 'characteristics in Objective 1, the women in the study were further categorised into three groups by BMI and ethnicity as non-obese (NOB; BMI$\leq$ 30 kg/m^2) black and NOB white women and obese (OB; BMI>30kg/m^2) black women and compared. Not having the OB group of white women available, for the effects of ethnicity in the collected variables, NOB groups of black and white women were compared, while the effects of obesity were assessed from the NOB and OB group of black women. A parametrical two-tailed t-test for independent groups (i.e. unpaired) was used in the analysis and p-values for differences are entered in Tables 3.2 and 3.4. Due to a significant age difference between NOB black and white women, the particular p-values are presented as age-adjusted.

The CST, CgA or CgA/CST associations with insulin, IR, HIC, DIo, IGI and with confounders (anthropometric and metabolic variables, the adiposity and inflammation markers) have been evaluated as univariate associations using Pearson correlation. Relationship with categorical variables (demographic variables and SES) was assessed by one-way ANOVA. The associations were evaluated for all women and then separately for black and white women groups (**Objective 2**).

Where the significant correlations of CST, CgA and of the CgA/CST ratio with the above variables were found (**Tables 3.5 - 3.7** in Results), these were further evaluated for independent associations using backward stepwise multiple linear

regression analysis, in which CST (CgA and CgA/CST) were tested as predictors. Each model was adjusted for BMI, age and other pre-selected confounders (**Objective 5**); final models are presented in **Table 3.8**.

The co-linearity between variables entering each model, was evaluated using variance inflation factor (VIF) as an inverted value of the Tolerance (t) given in Partial correlation menu of the multilinear regression for each variable. VIF equal or greater than 5 (or t=<0.2) was considered as an indicator of significant collinearity (Sheater, 2009). Such variables were removed or analysed separately and the model with higher AR^2 was taken as the result.

Catestatin, CgA and CgA/CST were highly collinear; therefore they entered the model subsequently, in repeated analysis, replacing each other. , to restrict the number of variables entering the models according to the number of participants (Petri, 1988), only six variables (for number of 67 black women) with the strongest correlation were tested at a time. The remaining variables were then analysed subsequently in replacement for the weakest correlated variables in the original model.

Pre-selection of confounders: Pearson's correlation analysis of the dependent variable with other variables was carried out. Significantly associated variables were pre-selected as potential confounders to enter the model (**Tables A1, B1, C1, Appendix A-C).** Each model was further corrected for BMI and age, family genetic disease background and family monthly income. The latter was re-coded to binomial form as ≤ R 40 000 and > R 40 000 for the analysis.

Reasoning for the removal of some significantly correlated variables: Next to VIF≥ 5, variables with very high correlation coefficient (R>0.800) were found to be all well-known predictors for the particular dependent variable or the part of its formula. For their strong relationship, they caused masking of the effects of other, less strongly associated variables.

2.12 Dealing with missing values

Unfortunately, in total 20 values of the IL-6 test were missing as the manufacturer discontinued the old kit (EL-H0102) and replaced it with a new kit (EL-H6156) with increased sensitivity during the study proceedings. Some samples were re-analysed but due to a restrained study budget, additional kit for all samples re-analysis could not have been purchased. To omit reduction in power due to case-wise deletion of values from the other variables during the analysis, all the original models were performed without IL-6 correction and only where the IL-6 correlated significantly with the dependent variable, this regression analysis was performed in addition with IL-6.

Only a minority of white women attended for the OGTT as they were enrolled in external enrolment points. For this reason, values for 30-minute glucose and insulin and derived indices IGI and DIo are available only for a few white participants (See Study Limitations). These values are displayed in descriptive tables. However, to omit a reduction in power due to case-wise deletion of values from the other variables, separate analyses were performed where indicated. The reduced numbers in these variables may bias the interpretation of the results. Similar number of black women and white women were compared in the BMI-split categories (Obese-OB and non-obese-NOB) for NOB group.

2.13 Ethics

The study has been approved by the Human Research Ethics Committee (medical; HREC) of the Faculty of Health Sciences of the Witwatersrand University (Ref No: M200922). The study ethics addendum was received from HREC for recruiting in external points (Path Care Vermaak-Pretoria and Wits Donald Gordon Medical Centre).

2.14 Funding.

The study has been funded by the NHLS Research Trust for the Development Grant, the NHLS K-funding and the Wits University research grant (Faculty Research funding - FRC).

Chapter 3

3. Results

This Chapter includes two major sections; first, the description of the participants' basic characteristics is given in the Descriptive section, including the demographic, anthropometric and metabolic characteristics for each ethnic study population group (Objectives 1, 2 and 3).

The demographic data collected from the enrolment questionnaire and the SES questionnaire are summarised graphically and evaluated for differences between groups. The basic anthropometric and metabolic characteristics are displayed in tables. These include basal insulin, HOMA-IR levels, CST, and CgA concentrations, and the CgA/CST ratio and their comparison between the ethnic groups (Objectives 2 and 3). Comparison tables split by BMI and ethnic were added for a better understanding of ethnic vs BMI effects.

The study Objectives 3 - 4 are the main subject of the Analytical part of the Results. The majority of results are presented for all participants and are separated by ethnicity. The CST (CgA; CgA/CST) effects on glucose–insulin dynamics regression analysis is conducted in black women only as no associations were found for white women.

3.1 Descriptive data: Characteristics of the study participants (Objective 1)

3.1.1 Demographic data:

The participating women ranged in age from 19 to 50 years with a median age of 29 (22; 34) years. Based on **Figure 3.1** below, the category 19 - <30 years included most of the participants including 66% of the black and 44% of the white study participants. The lowest representation of women was in the age category of 40 years and above with 7% and 24% for black and white women respectively (**Figure 3.1**).

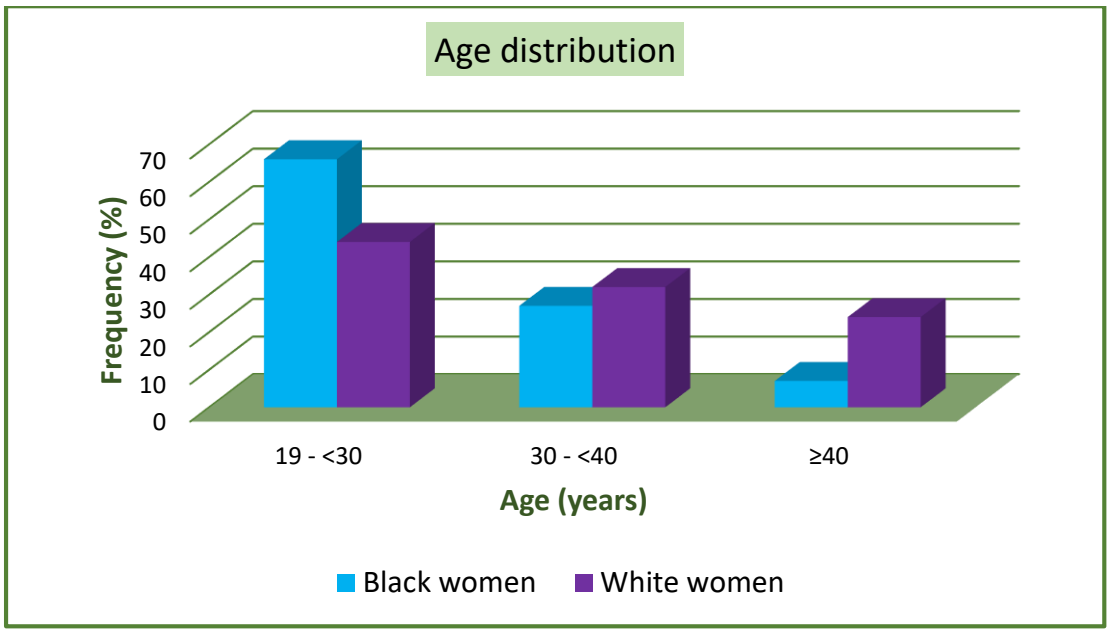


Figure 3.1: Distribution of enrolled women in age categories.

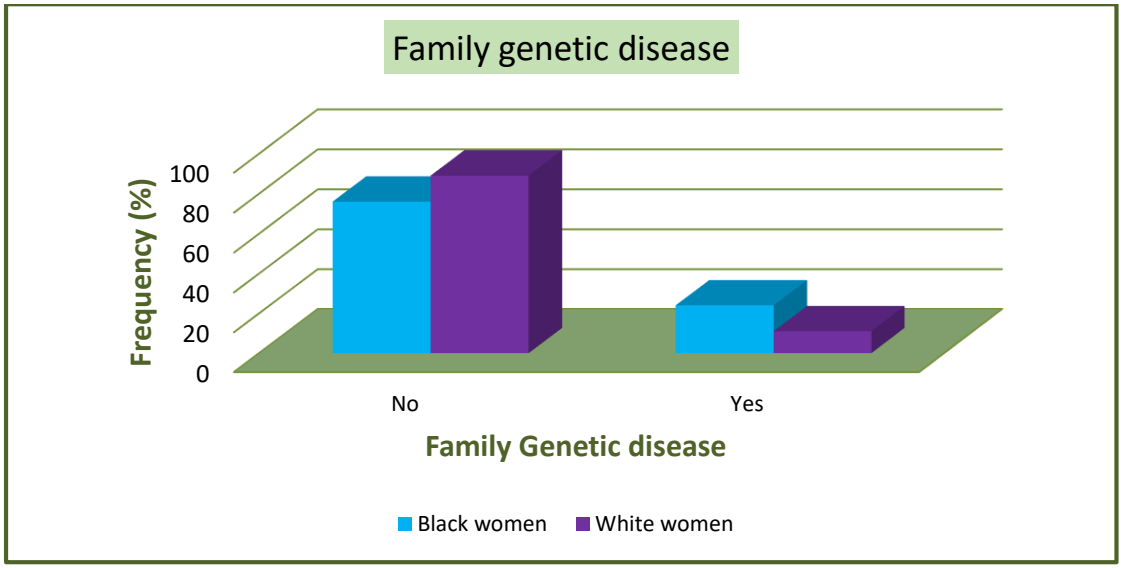


Figure 3.2: Representation of women with/without family history of genetic disease.

Only 18% of women had a family history of genetic disease distributed as 12% and 5.5% for black and white women respectively (**Figure 3.2**). There was no significant difference between groups (Chi^2 test (df 1, n=121) =3.28, p=0.070).

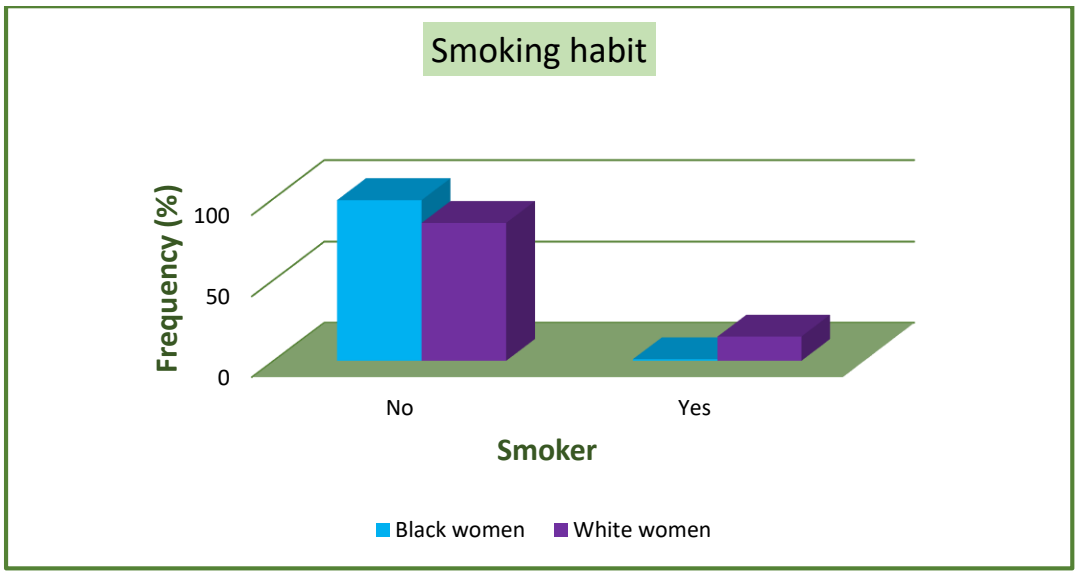


Figure 3.3: Representation of the smoking habit in the participants.

Most of the participants were non-smokers, with 0.5% of black women and 7.5% of white women being smokers as indicated in the Enrolment questionnaire (**Appendix F** and **Figure 3.3**). Due to this large difference between smokers and non-smoker on both sides, this variable has not been further used in the analysis.

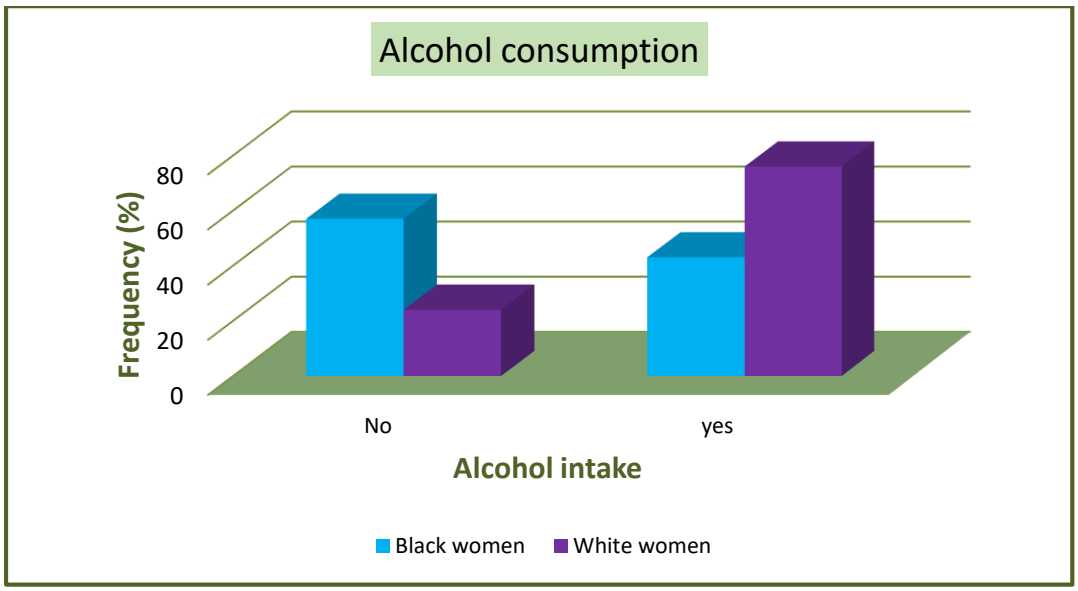


Figure 3.4: Alcohol consumption amongst the participants.

White women had a higher percentage of alcohol intake (76%) compared to black women (43%; **Figure 3.4**). This difference between groups was significant (Chi^2 test (df=1, n=121) = 13.07, p<0.001).

3.1.2 Socio-economic background:

All women in the study had matriculated. In addition, 91% of black women and 98% of white women had tertiary education.

There were more white women (54%) with a higher family monthly income (> R 60 000) compared to black women (12%). The majority of black women (30%) had a family income ranging from R 21 000 to R 40 000 per month as indicated in **Figure 3.5** below. The family income presented the major difference in SES between groups (Chi^2 test (df=2, n=121) =20.86, p<0.001), being higher in white women.

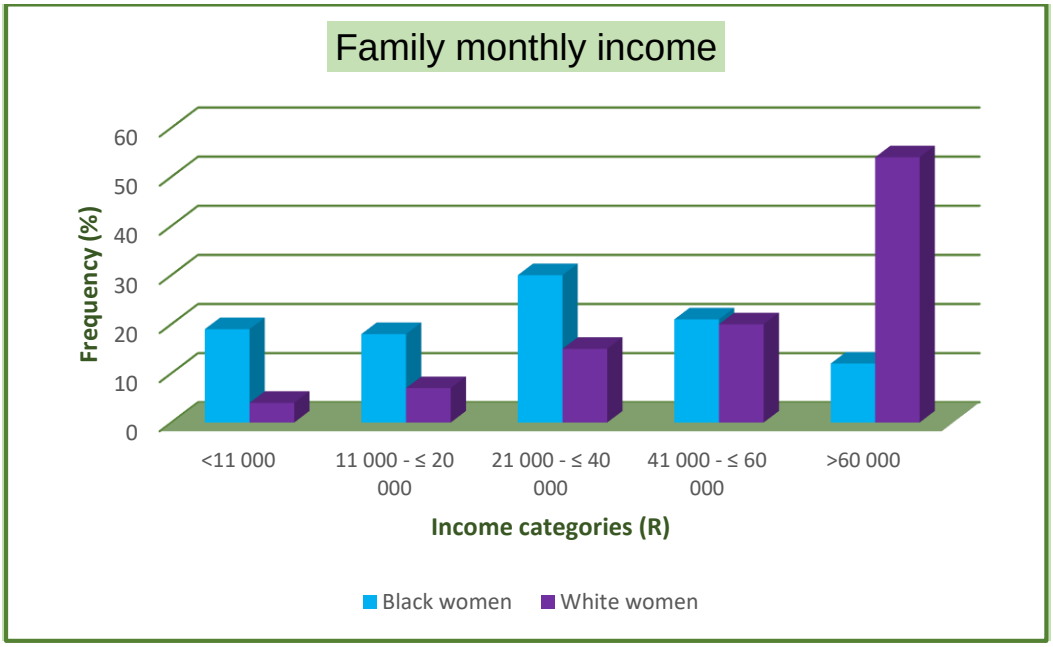


Figure 3.5: Family monthly income distribution for each ethnic group.

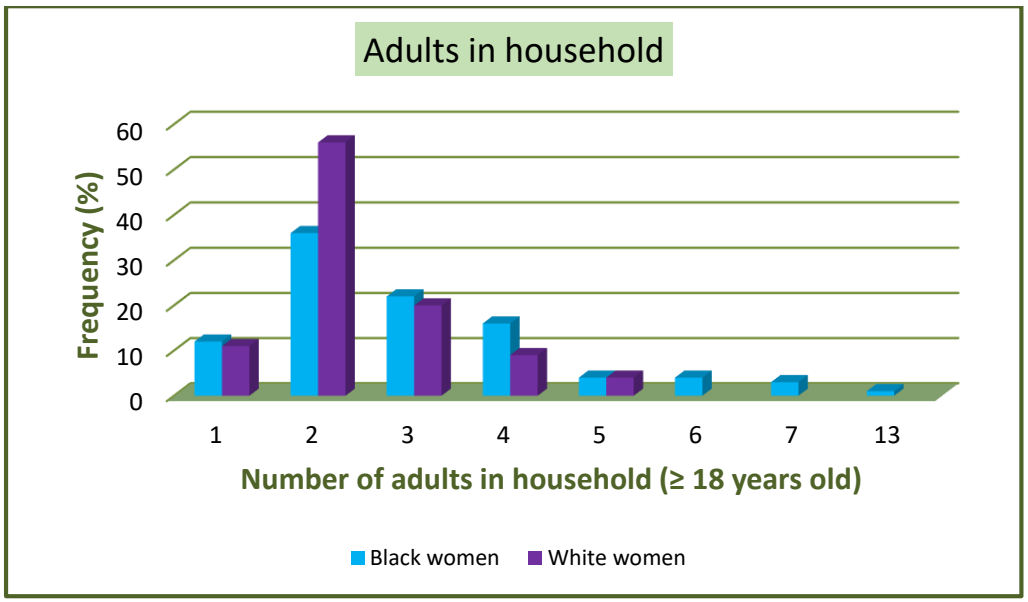


Figure 3.6: Number of adults in the household (≥18 years of age) for each ethnic group.

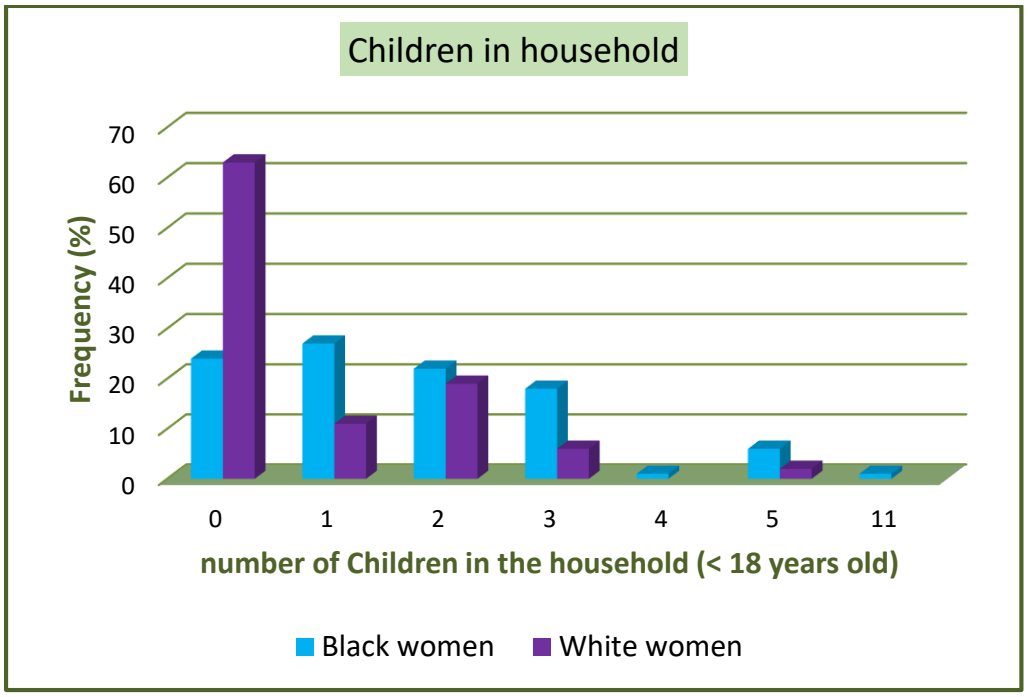


Figure 3.7: Number of children in the household (<18 years of age) for each ethnic group.

The number of adults and children in the black participant household was greater compared to the white participants. Over 60 % of white women (63%) had no children in the household, while only 24% of black women had no children in the household as indicated in the **Figures 3.6 and 3.7** above. Both variables were found

to have significant difference between groups (Chi^2 test (df=1, n=121) = 4.34, p=0.037 for the former and Chi^2 test (df=1, n=121) = 6.85, p=0.009 for the latter variable).

3.1.3 Characteristics of the study population – Anthropometric data

Table 3.1 below displays mainly participants' anthropometric characteristics and derived indices of adiposity. In the average, black women were significantly younger (26 (21; 32) years) than white women (30 (23.5; 35) years; p<0.001), shorter but heavier, with significantly higher BMI ((26.9 kg/m^2 (22.4; 31.4) kg/m^2 vs (23.5 kg/m^2 (20.8; 25.9) kg/m^2; p=0.001). All adiposity indices were significantly higher at black women. However, the groups were not BMI and age matched. Although an adjustment for these variables was done in a regression analysis and p-values are presented in the tables, for a better understanding of adiposity vs ethnic effects, tables splitting the data by BMI groups (non-obese, NOB and obese, OB) and ethnic were constructed and compared (**Tables 3.2, 3.4 a and 3.4 b**).

Black women in non-obese (NOB) and obese (OB) groups were of a similar age and height but OB women were heavier and had higher waist circumference and hip circumference (**Table 3.2**). In the BMI "matched" ethnic groups, black women had significantly larger WC 82.0 cm (77.4cm in NOB group and 96.8cm in OB group) than NOB white women (71.0 cm). White women were significantly older and taller. There was no difference between any groups in WHR and ABSI index. All women were matched for blood pressure and were normotensive.

Table 3.1: Age, anthropometric measurements and blood pressure for each ethnic group of the study participants.

Variables	Black Women (n=67)	White Women (n=54)	P-values
	Values expressed as mean± SD or median (IQR)		
Age (years)	26 (21; 32)	31 (24; 38)	<0.001
Height (m)	1.62 ± 0.07	1.66 ± 0.07	0.026
Weight (kg)	72.1 (57.2; 86.5)	63.4 (58.1; 72.7)	0.076
BMI (kg/m^2)	26.9 (22.4; 31.4)	23.5 (20.9; 25.9)	0.001
WC (cm)	82.0 (73.0; 92.0)	71.5 (66.0; 78.0)	<0.001
HC (cm)	111 (101;120)	98 (94; 105)	<0.001
WHR	0.75 (0.70; 0.80)	0.72 (0.69; 0.76)	0.002
ABSI ($m^{11/6}\ kg^{-2/3}$)	0.12 (0.11; 0.12)	0.11 (0.11; 0.12)	0.005
WHtR	0.51 (0.46; 0.57)	0.43 (0.40; 0.47)	<0.001
Systolic BP (mmHg)	120 (113; 128)	120 (110; 126)	0.440
Diastolic BP (mmHg)	74 ± 1	72 ± 7	0.250

SD: standard deviation, IQR: interquartile range, BMI: body mass index, WC: waist circumference, HC: hip circumference, WHR: waist to hip ratio; ABSI: body shape index, WHtR: waist to height ratio, BP: blood pressure

Table 3.2: Age and anthropometric measurements for each ethnic group with BMI categories.

Variables	Black Women BMI ≤ 30 kg/m² (NOB; n=46) (A)	Black Women BMI > 30 kg/m² (OB; n=21) (B)	White Women BMI ≤ 30 kg/m² (NOB; n=47) (C)	P-values (A-B)	P-values (A-C)
	Values expressed as mean± SD or median (IQR)			Unadjusted	Unadjusted
Age (years)	25 (21; 32)	28 ± 6.04	30 (23; 34)	0.395	0.004
Height (m)	1.62 ± 0.07	1.61 ± 0.07	1.67 (1.62; 1.70)	0.304	0.013
Weight (kg)	63.4 (55.2; 72.4)	93.0 ± 16.48	63.7 ± 9.40	<0.001	0.997
BMI (kg/m²)	24.1 (21.7; 27.0)	35.8 ± 4.15	23.0 ± 2.74	<0.001	0.073
WC (cm)	77.4 ± 9.61	96.8 ± 13.56	71.0 (66.6; 76.0)	<0.001	0.003
HC (cm)	105 (98; 111)	124 ± 9	97 (93; 104)	< 0.001	< 0.001
WHR	0.74 (0.69; 0.79)	0.78 ± 0.09	0.72 (0.69; 0.75)	0.097	0.264
WHtR	0.47 (0.44; 0.53)	0.61 (0.57; 0.66)	0.42 (0.40; 0.46)	<0.001	<0.001
ABSI	0.118 ± 0.012	0.113 ± 0.011	0.113 (0.109; 0.118)	0.086	0.095
Systolic BP (mmHg)	118.2 ± 10.9	123.6 ± 10.7	118.2 ± 10.4	0.086	0.591
Diastolic BP (mmHg)	73.0 ± 8.1	75.9 ± 7.8	72.1 ±7.1	0.160	0.990

SD: standard deviation, IQR: interquartile range, BMI: body mass index, WC: waist circumference, HC: hip circumference, WHR: waist to hip ratio; ABSI: body shape index, WHtR: waist to height ratio, BP: blood pressure; Obese (OB): BMI > 30kg/m², Non-obese (NOB): BMI ≤ 30kg/m²

3.1.4 Characteristics of the study population – Metabolic data

Table 3.3 summarises metabolic characteristics for each of the study ethnic groups. Although all participants were normoglycaemic, black women had significantly lower basal glucose levels (4.7 ± 0.3 mmol/L) compared to white women (4.8 ± 0.3 mmol/L , 30-minute glucose concentrations of the OGTT were significantly lower in black women independently of age and BMI.

There was no significant difference in fasting C-peptide between the two ethnic groups, although OB black women had significantly higher C-peptide than NOB black women (**Table 3.4**). The C-peptide results were compared with the available ranges utilised in the routine diagnostic laboratory. All participants except for two black women (one with high C-peptide of 1.72 nmol/L and one with low C-peptide of 0.30 nmol/L) had C-peptide within the reference interval of 0.36 nmol/L to 1.45 nmol/L.

Significant ethnic differences were observed for basal HIC and early glucose-stimulated insulin secretion (expressed as IGI). The HIC was lower whereas IGI was higher in black women as compared to the white participants (**Table 3.3**). The highest insulin clearance was observed in white NOB women (11.5 (9.8; 14.5) when compared to OB black participants while the insulin secretion was exactly opposite (but marginally non-significant (p=0.051); NOB white women had the lowest IGI and OB black women the highest level (**Table 3.4**).

Interestingly, the β-cell function, evaluated by DIo, was found to be higher in black women (**Table 3.3**). However, comparing between the BMI/ethnic groups, black OB, black NOB and white NOB women, no significant difference in DIo was observed (**Table 3.4**).

Metabolic indices of adiposity, leptin and adiponectin, were both significantly different between the groups, with leptin being higher and adiponectin lower in black women, independently of the age and BMI (**Table 3.3**). Black OB women showed about 3-fold higher leptin than black NOB women while white NOB women had almost 10-fold higher adiponectin concentrations than black OB women (**Table 3.4**). Inflammatory markers, MCP-1 and IL-6 were both higher in black women independent of age and BMI (**Tables 3.3 and 3.4**)

Table 3.3: Metabolic characteristics for each ethnic group.

Variables	Black Women (n=67)	White Women (n=54)	P-value (Un-adjusted)	P-values (BMI, age-adjusted)	Reference interval
	Values expressed as mean± SD or median (IQR)				
GLC-F (mmol/L)	4.7 ± 0.3	4.8 ± 0.3	0.010	0.013	< 5.6
GLC-30' (mmol/L)	6.3 (5.3; 7.0)	7.3 (7.0; 8.4) [a]	0.010	0.018	6.1 – 9.4
INS-F (pmol/L)	72.7 (53.3; 106.6)	56.5 (41.8; 71.3)	0.001	0.310	18 – 173
INS -30' (pmol/L)	730 (547; 930)	476 (417; 896) [a]	0.260	0.730	208 - 1597
C-PEP (nmol/L)	0.59 (0.53; 0.79)	0.66 (0.50; 0.79)	0.890	0.040	0.36 – 1.46
HOMA-IR	2.06 (1.45; 3.07)	1.69 (1.26; 2.16)	0.003	0.559	-
IGI (pmol/mmol)	447 (258; 773)	204 (122; 437) [a]	0.001	0.019	-
DIo (mmol/L)$^{-1}$	5.6 (3.9; 10.1)	3.1 (2.0; 7.2) [a]	0.046	0.016	-
ISI (1/HOMA-IR)	0.48 (0.33; 0.69)	0.59 (1.46; 0.79)	0.003	0.556	-
HIC molar ratio	8.2 (6.9; 10.5)	11.5 (9.5; 14.5)	<0.001	<0.001	-
CgA (ng/mL)	55.9 ± 20.0	51.0 ± 15.2	0.020	0.002	<100[#]
CST (ng/mL)	22.1 (15.0; 31.6)	33.0 (25.0; 59.5) [b]	<0.001	<0.001	-
CgA/CST	2.43 (1.63; 3.99)	1.33 (0.85; 2.18)[b]	<0.001	<0.001	-
Leptin (ng/mL)	5.8 (3.2; 11.5)	3.1 (1.5; 5.9)	<0.001	0.015	0.5 – 6.0[#]
Adipon. (µg/mL)	1.6 (0.8; 3.0)	9.3 (2.2; 11.2)	<0.001	<0.001	0.6 – 9.0[#]
IL-6 (pg/mL)	2.5 (1.9; 3.9)	1.0 (0.3; 2.1)[c]	<0.001	<0.001	1.2 – 4.5[#]
MCP-1 (pg/mL)	488 ± 173	470 ± 187	0.048	0.005	200 – 400[#]
GLC-120' (mmol/L)*	5.8 (5.3; 6.4)	6.2 ± 0.6[a]	0.014	0.035	<7.8

[A](n=13), [b](n=52), [c](n=46); [#]Kit manufacturer's expected reference intervals for the research kits.

*GLC-F: glucose fasting, INS-F: insulin fasting, C-PEP: C-peptide, GLC-30': glucose-30 minutes, INS-30'.: insulin-30 minutes, HOMA-IR: homeostatic model assessment for insulin resistance, IGI: insulinogenic index, DIo: oral disposition index, ISI: insulin sensitivity index, HIC: hepatic insulin clearance, CgA: chromogranin A, CST: catestatin, Adipon.: adiponectin, IL-6: interleukin-6, MCP-1; monocyte chemoattractant protein-1. *GLC-120': The values based on a finger-prick evaluation of glycaemia at 2h of the OGTT (not used in further analyses).*

Table 3.4(a): Metabolic characteristics of glucose – Insulin dynamics for each ethnic group by BMI categories.

Variables	Black Women BMI ≤ 30 kg/m^2 (NOB; n=46) (A)	Black Women BMI > 30 kg/m^2 (OB; n=21) (B)	White Women BMI ≤ 30 kg/m^2 (NOB; n=47) (C)	P-values (A-B)	P-values (A-C)	
	Values expressed as mean± SD or median (IQR)			Unadjusted	Un-adjusted	Adjusted for age
GLC-F (mmol/L)	4.6 ± 0.3	4.8 (4.6; 4.0)	4.8 ± 0.4	0.007	0.001	0.008
GLC-30' (mmol/L)	6.1 (5.2; 6.6)	6.4 ± 0.9	7.3 (5.8; 8.9)[a]	0.232	0.001	0.008
INS-F(pmol/L)	68.4 (47.5; 84.2)	92.8 (76.3; 157.0)	56.2 (41.8; 71.3)	<0.001	0.018	0.114
INS -30' (pmol/L)	673 (418; 906)	891 (759; 1344)	709 ± 501[a]	0.002	0.769	0.751
C-PEP (nmol/L)	0.61 ± 0.16	0.85 ± 0.35	0.63 (0.50; 0.76)	0.001	0.292	0.143
HOMA-IR	1.8 (1.3; 2.7)	2.7 (2.3; 5.0)	1.7 (1.3; 2.0)	0.001	0.089	0.299
IGI (pmol/mmol)	395 (229; 684)	655 (443; 1011)	303 ± 217[a]	0.019	0.061	0.051
DIo (mmol/L)$^{-1}$	6.16 (3.86; 12.96)	5.61 (4.09; 8.59)	6.07 ± 4.66[a]	0.978	0.229	0.383
ISI (1/HOMA-IR)	0.56 (0.37; 0.74)	0.34 (0.20; 0.43)	0.60 (0.50; 0.79)	<0.001	0.089	0.299
HIC (molar ratio)	9.2 ± 2.3	7.0 (6.5; 8.8)	11.5 (9.8; 14.5)	0.007	<0.001	<0.001

[a]*n=11,*

GLC-F: glucose fasting, INS-F: insulin fasting, C-PEP: C-peptide, GLC-30 min.: glucose-30 minutes, INS-30 min.: insulin-30 minutes, HOMA-IR: homeostatic model assessment for insulin resistance, IGI: insulinogenic index, DIo: oral disposition index, ISI: insulin sensitivity index, HIC: hepatic insulin clearance.

Table 3.4(b): Metabolic characteristics of glucose - Insulin dynamics for each ethnic group by BMI categories.

Variables	Black Women BMI ≤ 30 kg/m² (NOB; n=46) (A)	Black Women BMI > 30 kg/m² (OB; n=21) (B)	White Women BMI ≤ 30 kg/m² (NOB; n=47) (C)	P-values (A-B)	P-values (A-C)	
	Values expressed as mean± SD or median (IQR)			Unadjusted	Un-adjusted	Adjusted for age
CST (ng/mL)	19.5 (15.0; 25.7)	27.0 (15.4; 34.6)	36.1 (25.2; 62.8)	0.140	<0.001	<0.001
CgA (ng/mL)	61.5 ± 19.0	43.5 ± 16.7	51.4 (44.5; 58.7)	0.001	<0.001	0.005
CgA/CST ratio	2.96 (1.79; 4.09)	1.66 (0.78; 2.78)	1.32 (0.89; 1.96)	0.145	<0.001	<0.001
Leptin (ng/mL)	4.9 (2.4; 7.1)	15.4 ± 7.2	2.8 (1.5; 5.5)	0.001	0.002	0.002
Adiponectin (ug/mL)	1.69 (0.97; 3.30)	0.99 (0.63; 1.59)	9.59 (2.37;11.27)	0.049	<0.001	<0.001
IL-6 (pg/mL)	2.41 (1.76; 3.58)	3.25 ± 1.82	0.70 (0.19; 1.80) [b]	0.254	<0.001	<0.001
MCP-1 (pg/mL)	472.2 ± 173.3	522.71 ± 171.79	326.88 ± 157.67	0.265	0.001	<0.001
GLC-120' (mmol/L)*	5.50 (5.10; 6.20)	6.30 (5.70; 6.60)	6.20 (5.70; 6.90)	0.014	0.026	0.061

[b] *n=40*

*CgA: chromogranin A, CST: catestatin, Adipon: adiponectin, IL-6: interleukin-6, MCP-1; monocyte chemoattractant protein-1, *GLC-120: The values based on a finger-prick evaluation of glycaemia at 2-hours of the OGTT (not used in further analyses).*

3.1.5 Assessment of basal insulin and insulin resistant in both ethnic groups (Objective 2):

First, the basal insulin results were compared with the available ranges utilised in the routine diagnostic laboratory. All white women, except for one, had normal fasting insulin levels (7.9 mIU/L (5.8; 9.9) mIU/L), while among black women (10.1 mIU/L (7.4; 14.8) mIU/L), a small fraction (7%) exceeded the upper normal laboratory reference limit (2.6 – 24.6 mIU/L). These women were considered as hyperinsulinaemic.

Further, in unadjusted comparison, basal insulin and IR (as HOMA-IR) were significantly higher in black women which were less insulin sensitive, as ISI (as 1/HOMA-IR) was significantly lower. However, all this significance was lost after adjustment for BMI and age (**Table 3.3**). Meanwhile, NOB black and white women had comparable insulin (both fasting and 30-minute, HOMA-IR levels and insulin sensitivity, the OB black women showed significantly higher insulin (fasting and 30-minute) and HOMA-IR levels than NOB black women; **Table 3.4**).

3.1.6 Comparison of CST concentrations between groups (Objective 3).

Although no significant differences between the ethnic groups were identified in IR, they were found for CST levels, independently of the age and BMI of the participants. Black women had lower CST concentrations than white women (**Table 3.3).**

White NOB women had almost double CST concentration than NOB black women. Interestingly, black OB women showed slightly higher CST levels than black NOB women but this difference was not significant (**Table 3.4**).

Further, comparing levels of the CgA (prohormone CST), the opposite relationship was found, with higher levels observed in black women and so the CgA/CST ratio both independently of BMI and age (**Table 3.3**). Black NOB women showed the highest CgA and CgA/CST levels, while black OB women had the lowest ones of all three BMI/ethnic groups. The CgA values of the study participants were compared with the CgA kit manufacturer's reference interval cut-off value of 100 ng/mL

(evaluated in white European population of men and women). All women in the study, irrespective of their ethnic origin, had normal CgA concentrations, except for one white woman of a borderline CgA level.

3.2. Analytical Statistics

3.2.1 Evaluation of CST (CgA; CgA/CST) associations with variables of glucose-insulin dynamics (IR and surrogate indices), confounders (anthropometry, adiposity and inflammation markers) and demographic and SES variables (Objective 4).

For this task, the correlations of CST (CgA and CgA/CST) with age and the continuous metabolic and anthropometric variables were carried out. The results are presented for all participants irrespective of ethnicity and then for black women and white women separately (**Tables 3.5 and Table 3.6 and Table 3.7**).

The relationship between CST (CgA and CgA/CST) and categorical demographic and SES variables was assessed (See 3.2.1.4).

3.2.1.1 Catestatin correlations. CST did not produce many significant associations with the variables in the analysis (**Table 3.5**). For all the participants; CST showed a direct relationship with age, indicating that older participants were having higher CST. However, the association with age was not observed in any of the ethnic groups separately. Among black women, CST was mostly associated with anthropometric indices. The strongest direct relationship was found with WHtR, waist circumference and BMI. However, CST significantly negatively correlated with HIC and it had a positive association with leptin.

No significant correlations of CST with metabolic variables were found among white women; only a weak inverse association was noted with the anthropometric measurement of hip circumference. The CST correlated significantly negatively with the CgA/CST ratio in all women and both ethnic groups ($p<0.001$ for each).

Table 3.5: Significant correlations of CST with age, metabolic and anthropometric variables with corresponding correlation coefficients and p-values.

CST	All Participants (n=118)	Black Women (n=66)	White Women (n=52)
Age & Metabolic Variables:	Age (0.243; 0.008)	**HIC (-0.247; 0.045)**	
		Leptin (0.314; 0.010)	
	CgA/CST (-0.867; <0.001)	CgA/CST (-0.818; <0.001)	CgA/CST (-0.888; <0.001)
Anthropometric Variables:		BMI (0.330; 0.007)	HC (-0.269; 0.049)
		WC (0.331; 0.007)	
		HC (0.253; 0.041)	
		WHR (0.267; 0.030)	
		WHtR (0.348; 0.004)	

CgA/CST ratio: chromogranin A to catestatin ratio, HIC: hepatic insulin clearance, BMI: body mass index, WC: waist circumference, HC: hip circumference, WHR: waist to hip ratio, WHtR: waist to height ratio.

3.2.1.2 Chromogranin A correlations including all participants were driven by the correlations obtained for black women only, as no significant associations between CgA and the metabolic or anthropometric variables were found for the study group of white women (**Table 3.6**). In black women, CgA strongly negatively correlated with basal insulin) and HOMA-IR. Furthermore, CgA was negatively associated with basal C-peptide and insulin 30-minutes post glucose load levels and positively with HIC. A negative correlation was observed with adiposity marker leptin. From anthropometric indices, the strongest and negative association was found between CgA and BMI.

Chromogranin A correlated significantly positively with the CgA/CST ratio in all women and both ethnic groups (p<0.001 for each).

Table 3.6: Significant correlations between CgA and metabolic and anthropometric variables with corresponding correlation coefficients and p-values.

CgA	All Participants (n=118)	Black Women (n=66)	White Women (n=52)
Metabolic Variables:	**INS-F**(-0.261; 0.004)	**INS-F (-0.401; 0.001)**	
	C-PEP (-0.264; 0.004)	C-PEP (-0.325; 0.008)	
	HOMA-IR (-0.266; 0.004)	**HOMA-IR (-0.404; 0.001)**	
		HIC (0.366; 0.002)	
		INS-30' (-0.321; 0.009)	
	Leptin (-0.224; 0.015)	Leptin (-0.0362; 0.003)	
	CgA/CST (0.573; <0.001)	CgA/CST (0.636; <0.001)	CgA/CST (0.459; <0.001)
Anthropo-metric Variables:	BMI (-0.325; <0.001)	BMI (-0.442; <0.001)	No significance
	WC (-0.186; 0.043)	WC (-0.284; 0.021)	
	HC (-0.191; 0.038)	HC (-0.343; 0.005)	
	ABSI (0.194; 0.039)	ABSI (0.249; 0.044)	
	WHtR (-0.203; 0.027)	WHtR (-0.340; 0.005)	

INS-F: Fasting Insulin, C-PEP: C-peptide, HOMA-IR: homeostatic model assessment for insulin resistance, CgA/CST ratio: chromogranin A to catestatin ratio, INS-30': insulin-30 minutes, HIC: hepatic insulin clearance, BMI: body mass index, WC: waist circumference, HC: hip circumference, ABSI: body shape index, WHtR: waist to height ratio.

3.2.1.3 Chromogranin A/ CST ratio correlations. There were numerous correlations between the ratio and the other tested variables (**Table 3.7**). In all participants' group, the ratio was significantly inversely associated with age and basal C-peptide concentrations but with no anthropometry, although there was a

weak borderline significance with BMI (-0.179; p= 0.050 at alpha 5%). In black women, the CgA/CST ratio correlated significantly negatively with basal and early (30-minute) glucose-stimulated insulin, basal C-peptide, HOMA-IR and positively with HIC. A significant negative correlation was observed with leptin.

Table 3.7: Significant correlations between CgA/CST ratio and age, metabolic and anthropometric variables with corresponding correlation coefficients and p-values.

CgA/CST ratio	All Participants (n=118)	Black Women (n=66)	White Women (n=52)
Age & Metabolic Variables:	Age (-0.223; 0.014)	**INS-F (-0.418; <0.001)**	
		INS- 30' (-0.265; 0.030)	
	C-PEP (-0.213; 0.019)	C_PEP (-0.330; 0.006)	
		HOMA-IR (-0.408; 0.001)	
		HIC (0.387; 0.001)	
		Leptin (-0.461; <0.011)	
	CgA (0.573; <0.001)	CgA (0.636; <0.001)	CgA (0.459; <0.001)
	CST (-0.867; <0.001)	CST (-0.818; <0.001)	CST (-0.888; <0.001)
Anthropo-metric Variables:	No significance	BMI (-0.533; <0.001)	WHR (-0.326; 0.016)
		WC (-0.462; <0.001)	
		HC (-0.419; <0.001)	
		WHtR (-0.499; <0.001)	

C-PEP: C-peptide, CgA: chromogranin A, CST: catestatin, INS-F: Fasting Insulin , HOMA-IR: homeostatic model assessment for insulin resistance, CgA/CST ratio: chromogranin A to catestatin ratio, HIC: hepatic insulin clearance, BMI: body mass index, WC: waist circumference, HC: hip circumference, ABSI: body shape index, WHtR: waist to height ratio.

From the anthropometric variables, the strongest negative correlation of the CgA/CST ratio was found for BMI. However, waist circumference, hip circumference and WHtR showed a strong negative relationship. In white women, there was a

negative relationship with WHR. As expected, the CgAA/ CST ratio correlated significantly positively with CgA and negatively with CST in all women and both ethnic groups.

3.2.1.4 Evaluation of CST (CgA; CgA/CST) associations with lifestyle/ health factors and socio-economic status variables (Objective 4).

Variables tested in ANOVA: Family genetic disease, alcohol consumption, number of adults and number of children in a household and family monthly income. There were not many associations of CST with the categorical variables. For all women, CST levels were associated positively with family monthly income ($F=11.73$, $p=0.001$), indicating that CST levels were increased with higher living standard. For black women, there was a significant association with family genetic disease ($F=13.43$; $p<0.001$), indicating that women with the family history of genetic disease would have higher CST levels. There were no significant associations for CgA with any of the tested variables. CgA/CST ratio showed significant inverse relationship with family monthly income for all participants ($F=10.33$; $p=0.002$) and with the absence of family genetic disease ($F= 6.60$; $p= 0.13$) in black women.

3.2.2 Catestatin (CgA, CgA/CST) effects on insulin, insulin resistance and the indices of insulin-dynamics (Objective 5).

In the above correlation analysis, focussing on the variables of interest, CST correlated significantly with HIC and in black women. CgA correlated significantly with insulin fasting and HOMA-IR in all participants and/or black women and with HIC in black women only. Similarly, CgA/CST ratio correlated significantly with insulin fasting, HOMA-IR and HIC in black women only. Since no significant associations were found for IGI or DIo, and for white women, these were not assessed further. The correlations found for all women reflect rather associations found for black women only.

Therefore, in this task, effects CST (CgA and CgA/CST) on fasting insulin, HOMA-IR and HIC in black women were investigated only.

3.2.2.1 Objective 3, part A: Selection of the confounders. A univariate correlation analysis was performed for fasting insulin, HOMA-IR and HIC with other variables to identify potential confounders to be entered into the regression model next to CST (CgA or CgA/CST ratio; **Tables, Appendix A-C; Chapter 2.11.2**).

From the significant correlations, following confounders were selected for the analysis:

Analysis 1: Fasting insulin (dependent variable);

Chromogranin A (or CgA/CST ratio) predictor variables;

- Fasting glucose, HIC, DIo, leptin, adiponectin, BMI (with the strongest correlation among the anthropometric variables), family genetic background, family monthly income.
- Variables removed: HOMA-IR (VIF 6.1); C-peptide highly correlated with fasting insulin; 30-minute glucose- and 30-minute insulin considered as unlikely predictors of basal INS.
- Two models presented in **Table 3.8,** one including HIC (although R>0.800, HIC addition did not change the variables in the resulting model) and one without HIC.

Analysis 2: HOMA-IR (dependent variable)

Chromogranin A (or CgA/CST ratio) predictor variables;

- Fasting glucose, HIC, DIo, leptin, adiponectin, BMI, family genetic background, family monthly income.
- Variables removed: fasting insulin (VIF 5.3); 30-minute glucose and 30-minute insulinunlikely predictors of basal HOMA-IR; C-peptide (R=0.889);
- Two models presented in **Table 3.8,** one including HIC (although R>0.800, HIC addition did not change the variables in the resulting model) and one without HIC.

Analysis 3: Hepatic Insulin Clearence (dependent variable)

Catestatin (or CgA or CgA/CST ratio) predictor variables;

- Fasting glucose, DIo, leptin, BMI, age, family genetic background, family monthly income.
- Variables removed: fasting insulin, C-peptide (parts of the formula); 30-minute glucose and 30-minute insulin unlikely predictors of basal HIC; HOMA-IR (R=-0.804).
- Two models presented in **Table 3.8,** one including age and one without age correction.

3.2.2.2 Objective 5: Regression analysis. Hepatic insulin clearance was regressed on catestatin, CgA and CgA/CST ratio (with replacement), and, further, fasting insulin and HOMA-IR were regressed on CgA and CgA/CST in separate multilinear models (**Table 3.8)**, correcting for the selected confounders.

No association of CgA or CgA/CST ratio with fasting insulin or HOMA-IR after correcting for BMI and other confounders was identified. Irrespective which of these two predictors was tested in the model, the outcome was the same for both.

Fasting insulin (as a dependant variable) had significant negative association with HIC, adiponectin and DIo and a positive association with BMI and fasting glucose. After removal of HIC (the strongest predictor) from the model, the rest of the variables remained, only their explanatory strength was reduced, as indicated by the AR^2 coefficient. Similar associations were obtained with HOMA-IR as a dependent variable; (**Table 3.8)**.

Catestatin produced significant correlation with HIC only (3.2.1.1., **Table 3.5**). However, after correcting for other variables and selected confounders, no such association was observed in the current analysis. The CgA or CgA/CST ratio was not significantly associated with HIC after correcting for other predictors and confounders. However, after removal of age from the predictors, CgA gained positive significance with HIC. Correcting for age and BMI only (DIo removed as a strong predictor), CgA/CST gained positive significance with HIC in the model. Among other predictors, HIC had significant positive association with DIo and age and negative with BMI. Removing age variable, CgA replaced age and leptin replaced BMI. No associations were found with family genetic disease or family monthly income in any of these models.

Table 3.8: Multivariate linear regression models for fasting insulin, HOMA-IR and HIC with CST (CgA or CgA/CST) as predictors, adjusted for selected confounders.

Dependant Variables	Independent variables with standardised β (β) coefficients, corresponding p-values and adj. R^2 with p-value for the full model. Black Women (n=65)		
	Corrected for all selected variables	**Corrected for reduced variables**	**Removed variables**
INS-F	HIC (-0.605; <0.001)	-	HIC
	DIo (-0.135; 0.043)	DIo (-0.373; <0.001)	
	Adipon. (-0.153; 0.014)	Adipon. (-0.192; 0.038)	
	GLC-F (0.236; 0.001)	GLC-F (0.269; 0.007)	
	BMI (0.175; 0.011)	BMI (0.366; <0.001)	
	AR^2 =0.791, p<0.001	**AR^2 =0.525, p<0.001**	
HOMA-IR	HIC (-0.561; <0.001)	-	HIC
	DIo (-0.125; 0.043)	DIo (-0.346; <0.001)	
	Adipon. (-0.143; 0.013)	Adipon. (-0.180; 0.037)	
	GLC-F (0.353; <0.001)	GLC-F (0.384; <0.001)	
	BMI (0.163; 0.011)	BMI (0.340; <0.001)	
	AR^2=0.820, p<0.001	**AR^2=0.591, p<0.001**	
HIC	DIo (0.460; <0.001)	DIo (0.395; <0.001)	Age
	BMI (-0.519; <0.001)	Leptin (-0.256; 0.034)	
	Age (0.468; <0.001)	CgA (0.239; <0.001)	
	AR^2=0.516, p<0.001*	**AR^2=0.314, p<0.001**	
	DIo (0.460; <0.001)	CgA/CST (0.300; 0.009)	DIo
	BMI (-0.519; <0.001)	Leptin (-0.296; 0.012)	
	Age (0.468; <0.001)	Age (0.376; <0.001)	
	AR^2=0.516, p<0.001*	**AR2=0.378; <0.001**	

GLC-F: fasting glucose, INS-F: fasting insulin, C-PEP: C-peptide, HOMA-IR: homeostatic model assessment for insulin resistance, DIo: disposition index, HIC: hepatic insulin clearance, Adipon: adiponectin, BMI: body mass index.

Chapter 4

4. Discussion

Ethnic disparities in IR between black women of African ancestry and their white counterparts were reported worldwide (Hasson, et al., 2015; Goran et al., 2002) and locally in South Africa (Van der Merwe et al., 2000; Goedecke[a] et al., 2009), affecting negatively black women. Although obesity plays a major role in the pathogenesis of IR, the ethnic differences are apparent in BMI controlled studies and in non-diabetic individuals (Haffner et al., 1996) indicating other factors may be involved.
Animal-based and *in-vitro* studies demonstrated the involvement of CgA and its cleavage products CST and PST in the regulation of glucose metabolism. The current study aimed to investigate, whether CgA-derived peptide CST (or their ratio CgA/CST - or CgA itself) may have a role in IR disparities between South African black and white women through its possible regulatory effects on IR or on other steps of the glucose-insulin dynamics.

Based on the measurements and questionnaire-collected data, we evaluated the demographic, socio-economic and anthropometric characteristics of the participating women compared them between groups and with available data from similar local studies. Further, we evaluated, whether the women differed in insulin resistance (assessed from the HOMA-IR index) or in any of the glucose-insulin regulatory steps. For this, we used OGTT-derived surrogate indices for early insulin secretion (insulinogenic index, IGI), β-cell function (oral disposition index, DIo) and the hepatic insulin clearance (molar ratio of fasting C-peptide to fasting insulin) and compared between groups and with the available literature. Data was collected for potential confounders, namely adiposity markers (leptin and adiponectin) and inflammation markers (IL-6 and MCP-1). The levels of CST (CgA and the ratio) were compared between groups; there is a lack of human studies involving CST in this field and the available kits are not standardized. The correlation analysis of CST (CgA and the ratio) with other collected data and confounders served to selection variables for the regression analysis, where association of CST (CgA and the ratio) with IR and indices of glucose-insulin dynamics was evaluated, correcting for anticipated confounders.

4.1 Study enrolment

The study included “apparently healthy”, normotensive and normoglycaemic urban black and white women from Parktown-Johannesburg and Centurion areas in the Gauteng province of South Africa. They were enrolled among the students and staff of the Wits and the associated Wits Donald Gordon Medical Centre, the NHLS and the Path Care staff. In total, 121 participants, including 67 black and 54 white women, completed the study.

The enrolled ethnic groups were not matched for BMI and age, as there was a lack of white obese women among the participants (see Study Limitations). For this reason, the descriptive statistics tables show the comparison of black women vs white women characteristics in two forms; unadjusted and BMI- and age-adjusted p-values. For a better understanding of the effects of ethnicity vs. obesity, the ethnic groups were split by BMI of 30 kg/m^2 into non-obese (NOB) and obese (OB) groups (excluding OB white women) and compared. Regression analysis included entire ethnic group with correction for BMI.

4.2 Demographic data and socio-economic status evaluation

In our study, the largest differences were noted in family monthly income and alcohol consumption, both being significantly higher in white women. Although only 18% of women had family history of a genetic disease, 2/3 of the genetic diseases were observed in black participants. This was significantly different. More white women were smokers and consumed more alcohol (76%) when compared to black women. Although the white participants consisted mostly of students, they had a higher family monthly income than the black participants, which consisted mostly of employed women. On the other hand, black participants had more adults and children in their household than their white counterparts. This could explain why the white women afforded to allocate more money for entertainment (e.g. buying alcohol) while black women had more household members to share the particular, in average lower, income with.

The demographic and SES data was re-coded for a comparison using 2x2 or 3x2 contingency tables due to very low numbers in some categories. For the regression analysis, only the significantly associated variables (family genetic disease and family income) were selected as confounders. Catestatin (and CgA/CST ratio inversely) correlated significantly with “family genetic disease” in black women and with “family monthly income” in white women. However, both variables left the regression models with insignificant effects.

4.3 Characteristics of the study participants

4.3.1 Age and anthropometry

The white participants were older on average, when comparing between BMI matched groups of black and white women. For this reason, all further analyses were adjusted for age. White women were taller.

Participating black women were heavier with higher BMI (within the overweight range), while white women were mostly within the normal BMI category. The BMI of the women in the matched NOB ethnic groups was normal (<25 kg/m^2) on average. However, the black women had higher waist circumference and hip circumference (but not WHR) when compared to white women. waist circumference is an indicator of central obesity and a proxy measure of visceral fat (VAT) and it is used in the detection of metabolic syndrome (MetS), which is known to have a higher prevalence in black South African women than in European population (Gradidge and Crowther, 2017).

The cut-off points for waist circumference associated with higher risk of MetS differ by ethnicity. Comparing these values with the published waist circumference cut off 92 cm for South African black women and 80 cm for European white women (Crowther and Norris, 2012), the OB group of black women has exceeded this cut-off level, although being normotensive and normoglycaemic. The NOB-black women and NOB-white women were below their particular cut-offs. However, since the WHR ratio did not significantly differ between groups (NOB black vs NOB white or NOB black vs OB black), it could be explained by a higher contribution of gluteofemoral fat (for which hip circumference is a proxy measure). Due to increasing contribution of

protective gluteofemoral fat with increasing BMI, the waist circumference cut-off levels for MetS detection increase (Manolopoulos et al., 2010; Gradidge et al, 2022). Since waist circumference and hip circumference have inverse relationship with the MetS risk, WHR might be a better indicator.

Different cut-off values for anthropometry measures and various indices for Sub-Saharan African women have been suggested (Gradidge et al, 2022), according to a low (<32.8 kg/m2) or a high (≥ 32.8 kg/m2) BMI. Comparing these cut-off levels (that included waist circumference, hip circumference, WHR, WHtR and ABSI), all NOB black women were well within the reference interval and OB black women were near the cut-off level but did not exceed it. This indicates, that both the white women and black women participants, based on these criteria, were metabolically healthy.

4.3.2 Metabolic variables

All study participants had normal basal glucose levels based on the selection criteria. These glucose concentrations were significantly lower in black women than in white women, although black women had significantly higher BMI. The basal glucose levels were significantly lower in BMI matched black vs white women. These glucose concentrations were comparable with the levels of black and white South African women reported by Keswell et al. (2016), although both ethnic groups in their study had larger BMI than the women in the current study. The concentrations of the 30-min OGTT-post load levels did not differ between BMI groups of black women but were higher in NOB white women. All black women completed OGTT and were verified as normoglycaemic. Glucose taken at 2-hours of the OGTT by a finger prick was comparable between BMI-matched ethnic groups but it was significantly higher in OB vs NOB black women.

Except for two black women with marginally higher and lower levels, the C-peptide concentrations were all within a normal laboratory range. Both ethnic groups matched for BMI had comparable concentrations (**Table 3.4**). However, significantly higher levels were seen in OB (vs NOB) black women, indicating increased C-peptide (and insulin) secretion at the basal state in this group. This differs from the study of Reimann et al (2007), who investigated ethnic differences in C-peptide and non-esterified fatty acid metabolism in pre-menopausal South African and white

European women with and without abdominal obesity. They found no difference in IR but the basal C-peptide levels were significantly higher in lean black women than in lean white women, while no difference between black and white women with abdominal obesity was seen. C-peptide in that study was significantly directly associated with triglyceride levels.

From three BMI/ethnic groups, the early insulin response to glucose challenge evaluated by IGI was the highest in OB black women. Marginally insignificantly higher IGI was seen in NOB black women in BMI matched and age adjusted comparison with white women. Comparing with the study of Goedecke[b] et al (2009), where both the normal weight and obese groups were significantly higher than their white ethnic BMI matched counterparts, the current study shows less observed difference between NOB white and black women (30% vs. 137% in the comparison study) and a greater difference among BMI groups of black women (66% vs. 16% in the comparison study). Their study included BMI matched South African apparently healthy black and white urban women.

Insulin response adjusted for the particular insulin sensitivity was evaluated by DIo, and interpreted as β-cell function and the glucose-disposal ability of the body (Chapter 1.1.4) (Cobelli et al., 2007). First, DIo was found significantly higher in black women (**Table 3.2**). However, comparing between the BMI/ethnic groups, black OB, black NOB and white NOB women showed no significant difference in DIo between any of these groups (Table 3.4). This discrepancy may have been caused by a bias from a small number of OB white women contributing to the overall ethnic comparison and generally by the low number of white women in this analysis. However, the latter finding is in agreement with the study of Goedecke[a] et al. (2009), where no significant difference between BMI and ethnic groups in DIo was detected.

Among the surrogate indices for glucose-insulin dynamics, women in the current study showed the highest significant difference in basal HIC. The highest clearance of insulin in the liver was observed in white NOB women and the lowest in black OB women; In detail, HIC between the BMI-matched ethnic groups was approximately 20% lower in black women, while obesity among black women accounted for a 24% reduction in HIC. This is in agreement with the previous reports on reduced HIC observed in black women with African ancestry, especially in those with an increased

risk for T2D (Bergman et al, 2019). This same pattern was noted already in children, adolescents or non-diabetic individuals (See Chapter 1.3). Osei and Schuster (1994) showed that steady-state HIC (based on the same HIC derived from the OGTT) was 33% lower in black vs white Americans. Piccinini et al. (2017) reported that hepatic and not extra hepatic insulin clearance is reduced in African American vs European apparently healthy normoglycaemic pre-menopausal women, indicating a primary role for the HIC in the maintenance of insulin sensitivity and the pathogenesis of T2D in the African-Americans (Bergman, 2019). The current study data on the local South African population support this.

The adiposity marker leptin was significantly higher in black women than in white women. In the comparison across the ethnic groups, black women had about 5.5-fold higher leptin concentration than white women. With increased obesity in black women, leptin concentrations tripled in OB group. Higher leptin levels in obese group were expected as they are associated with adiposity (Paz-Filho et al., 2012).

In contrary to leptin, adiponectin (anti-inflammatory cytokine) was significantly lower in black women. In the OB black women, the adiponectin concentrations were the lowest, accounting for almost 10-fold difference, when compared to NOB white women. This would be expected as the adiponectin blood levels are known to be inversely associated with obesity, therefore, lower in obese than in lean individuals (Lihn et al., 2005). This is in agreement with the study of Van Zyl et al. (2016), where the authors investigated adipokines in South African urban women from the Free State province. The obese African women had significantly lower adiponectin and higher leptin levels than lean African women. However, the authors did not investigate ethnic differences. Schutte et al. (2007) showed that normal weight African women had marginally lower adiponectin levels than their white counterparts. No ethnic difference was found for overweight or obese women in their study.

Inflammatory markers MCP-1 and IL-6 were both elevated in black women with the highest concentrations in the OB group. NOB white women had the lowest levels of these inflammatory markers. African-PREDICT study (Kriel et al., 2017) investigating cardiovascular disease and hypertension in apparently healthy young men and women, reported significant ethnic differences with higher MCP-1 levels in black women vs. white women. Serum IL-6 concentrations are positively linked to obesity

and visceral adipose tissue; therefore, they are significantly higher in obese individuals (Park et al., 2005) and correlate with BMI and other anthropometric measures of adiposity. As discussed earlier, although BMI matched, NOB black women in the current study had significantly higher waist circumference and hip circumference than NOB white women. This could be, at least partially, explained by the observation of Joffe et al. (2014) who identified relationship between IL-6 polymorphisms and measures of obesity and serum lipids in both black and white SA women.

However, the IL-6 results in this study have to be interpreted with caution due to a number of missing values (see the Study Limitations).

4.4 Insulin and insulin resistance comparison between groups:

(Objective 2)

The study further investigated whether the sampled population of the normoglycaemic and normotensive black women will present with the literature-anticipated characteristics of hyperinsulinaemia and higher IR in BMI matched comparison with white women.

However, in this study, only a small fraction of black women presented with hyperinsulinaemia was identified where basal insulin concentrations exceeded the upper normal laboratory range. Although differences between both ethnic groups were significant for fasting insulin and HOMA-IR in direct comparison, these were abolished by adjustment for BMI and age. Only differences attributable to obesity were associated with significantly higher insulin and HOMA-IR among black women. This finding is similar as in the study of Keswell et al. (2016) which included black and white South African urban women of a similar age and apparently healthy as the current study. Both ethnic groups in their study showed no significant difference in basal insulin and HOMA-IR levels (after adjustment for age and fat-mass-index) and black women had lower glucose concentrations.

In the study conducted by Goedecke et al (2009), no ethnic differences in DIo were identified as insulin response on the background of decreased insulin sensitivity in black women was sufficient to maintain normoglycaemia. In the current study, there

were no ethnic differences found for β cell function as there were no significant ethnic differences in insulin sensitivity (ISI or 1/HOMA-IR) observed, although IGI was marginally higher comparing across NOB BMI groups. However, a significantly reduced ISI (higher HOMA-IR) was seen with obesity in black women. Adequate compensatory early insulin response (IGI) maintained normoglycaemia and led to comparable DIo in both BMI groups of black women.

4.5 Evaluating difference in CST (CgA and CgA/CST) levels: (Objective 3)

Although no significant ethnic difference in IR was observed in this study, the main attention was still placed on the evaluation of CST levels in the study participants.

As hypothesized, black women showed significantly lower CST levels and this relationship retained its significance even after BMI and age adjustment. Interestingly, this effect was attributable to ethnic difference only, and no significant changes in CST between BMI groups were identified, although CST has anti-obesity effects (see Chapter 1.6). Obese children and adolescents had lower serum CST levels than healthy controls (Herold et al., 2021). The white women with highest CST levels were in NOB group and the black women with the lowest CST levels were not obese. The OB black women had only insignificantly higher CST levels than the NOB groups. Among the tested variables, a similar pattern had only fasting and 30-minute OGTT-stimulated glucose.

To our best knowledge, this is the first report showing ethnic differences in CST, specifically between black and white South African urban normoglycaemic and normotensive women.

Since this difference is independent of obesity, other unknown factors not measured in this study may apply and a genetic involvement in the CST ethnic differences cannot be excluded. Circulatory CST levels can be heritable, as documented previously on twin studies (O'Connor et al., 2008) and different genetic variants of CST were reported in different populations across the world. e.g., in Indian population, Ser-364 allele was strongly associated with higher plasma triglyceride and glucose levels but with significant decrease in plasma norepinephrine/

epinephrine levels (Sahu et al., 2012). No data on genetic variants of CST were reported in South Africa (see Follow-up research).

The serum CST levels reported in the literature are mostly associated with hypertension and cardiology field, and only one article reported CST values using the same CST kit (Wołowiec et al., 2020) as the current study did. (Currently, there are not many commercial human CST kits available and these kits are not standardized between manufacturers.) The population in the comparison included 52 Caucasian patients, mostly males, with various cardiac pathologies but hemodynamically stable and a control group. In both groups, CST levels declined after physical exertion but were generally lower in patients than in controls. The study group and the control group were not matched for age or BMI. The current study participants showed CST concentrations similar or higher (in white women) than the controls before the exercise (IQR 14.75-22.20 ng/mL) in the comparison study. The participants in the current study were rested before the sample collection.

With an opposite pattern, CgA was significantly higher in black women. In detail, CgA was the highest in NOB black women and significantly lower in the BMI matched white women. Interestingly, the lowest CgA concentrations were seen in OB black women and this reduction in CgA against the NOB black women was highly significant. The expression of human CgA gene (CHGA) is heritable (Mahapatra et al., 2005).

Low CST levels could result from a dysregulated CgA cleavage, which would present as a relatively increased CgA/CST ratio. In the current study, the CgA/CST ratio was significantly higher in black women, as hypothesized. Although the CgA/CST ratio levels followed the pattern of CgA distribution between BMI/ ethnic groups, the difference attributable to obesity (NOB vs OB black females) was not significant, as in case of CST.

The CgA values of the study participants were compared with the CgA kit manufacturer’s reference interval cut-off value of 100 ng/mL (evaluated in white European population). Irrespective of the ethnic origin and significantly higher CgA

levels in black women, all study participants had “normal” CgA concentrations, except for one white woman of a borderline CgA level. This has not been reported before. The kit is routinely used in the detection of neuroendocrine tumours, however, various metabolic and other health conditions can elevate CgA levels above the cut-off point. This proves that the women in the study were metabolically healthy, as requested by the study selection criteria.

ChromograninA is co-stored and co-released with catecholamines from adrenal medulla and has previously been suggested as a possible marker of stress. Plasma CgA level reflects the sympathetic tone and adrenomedullary system activity. Salivary CgA levels were associated with anxiety and depression. Long-term work stress was associated with high CgA levels and high CgA/CST ratio but not with CST (Liu et al., 2021).

4.6 Association of CST (CgA and CgA/CST) with insulin resistance and the indices of insulin dynamics (Objective 4; Tables 3.5-3.7).

Based on the correlation analysis, CST did not show any direct association with IR, as anticipated. The reason could be that no significant differences in HOMA-IR were found between both ethnic groups but indirect effects may be possible, e.g. via its interaction with leptin (see below). Leptin is involved in the regulation of glucose metabolism and insulin sensitivity; it reduces insulin synthesis and secretion and increases HIC. Leptin concentrations are associated with IR in both normoglycaemic and diabetic individuals independently of weight and adiposity (Paz-Filho et al., 2012).

Among all participants, CST correlated positively with age. This could reflect higher CST levels found in white females, because they were significantly older than black females. However, CST in white females failed to show any significant correlation, except for the negative correlation with HC (a proxy measure of lower body fat) and an expected negative association with the CgA/CST ratio. In experiments with CST-KO rodents, the positive effects of CST supplements on glucose regulation were

noted only in obese mice and did not affect glucose regulation in lean controls (Ying et al., 2018), indicating that CST acts only on obese models.

In black women only, CST correlated significantly negatively with HIC. This is an interesting finding as reports based on animal studies (see Chapter 1.6) suggested regulatory role of CST in HIC. The OB black women in the study had insignificantly higher CST levels than the NOB black women, while having significantly lower HIC. In comparison of NOB ethnic groups, CST levels were lower and so the HIC was lower in black women vs. white women. Inversely, this applied to the CgA/CST cleavage, which was highest in white females and lowest in the BMI matched black females. Obese black females had, again, insignificantly lower CgA cleavage so they yielded with slightly higher CST levels than NOB black females. It is possible that the lack of significance in CST elevation in OB groups could just be due to statistically low numbers (21) in this group and a large study would need to prove this effect.

The inverse correlation between CST and HIC found in the current study would support the hypothesis of Borges et al. (2013) for CST to be rather enhancing the HIC. This would correspond with white women having higher CST levels and higher HIC and vice versa. However, our data cannot give a clear answer as in the model corrected for BMI, age and other confounders; the CST effect was lacking significance.

Catestatin could be involved in the regulation of HIC indirectly, via its other effects (e.g. on obesity and hypertension) as pleiotropic hormone.

Our results show a significant positive correlation with leptin in black women, and CST-leptin interaction has been described previously in the literature. The CST anti-obesity effect is associated with its stimulation of leptin-induced signalling (via leptin receptor) in adipose tissue and inhibition of alpha-2 receptor signalling (Bandyopadhyay et al., 2017; See **Table 1.1**). Catecholamines regulate fat cells by balancing their action on adrenergic receptors β 1-3 (lipolytic effect) and α-2 (opposing effect) (Bandyopadhyay et al., 2012). Repeated long-term stress increases plasma catecholamines and their action desensitizes β adrenergic receptors and inhibits lipolysis and may prevent inhibition of leptin by catecholamines, leading to higher leptin levels (Bandyopadhyay et al., 2012). Long-

term elevated leptin levels, in turn, may desensitize leptin receptor and lead to obese phenotype.

Long term higher levels of stress including traumatic events, social stressors and psychological distress were reported in post-apartheid South African black population (Harriman et al., 2021) but among African American women with lower SES (Childs, 2020). Catestatin is a potent endogenous inhibitor of catecholamine secretion (Mahata et al, 2010). In a rodents' study (Bandyopadhyay et al., 2012), CST administered to CgA-KO (and so CST lacking; obese on regular chow diet) mice restored AR and leptin receptor sensitivity by reducing levels of catecholamines and further of leptin and significantly reduced adiposity. Other associated effects of CST are improvement of insulin sensitivity and normalisation of insulin and glucose levels (see **Table 1.1**).

Expression of leptin depends on insulin levels and glucocorticoids and catecholamines. Pancreastatin, the CgA cleavage product derived from pancreatic alpha-cells which opposes to CST effects, temporarily inhibited leptin expression and secretion in rat adipocytes (Gonzales –Yanes and Sanchez, 2003). Therefore, the dysregulated CgA cleavage with lower CST and higher PST can contribute to the above regulation.

Correlation of CgA with fasting insulin and C-peptide can reflect the CgA regulatory function in the β-cell secretory granules, including the proinsulin cleavage to insulin and C-peptide during the process of the granules' maturation and their co-secretion with other secretory products, including CgA and CST (see Chapter 1.5). However, other granins are involved in some steps of this regulation e.g. chromogranin B in the regulation of insulin secretion (Herold et al., 2021).

Correlations of CgA and of the CgA/CST ratio with leptin are inverse to those seen for CST and they might be reflecting CST associations with leptin (See Chapter 4.5). And as above already mentioned, a work stress affected their levels more than of CST (Liu et al., 2021).

Chromogranin A and the CgA/CST ratio positively correlated with HIC**.** This association remained significant in HIC predictors' model, independently of leptin and DIo for CgA, and of leptin and age for the CgA/CST ratio. However, the significance was apparent only when very strongly associated and wellknown predictors were removed from the model (as explained at the beginning of Chapter 4.6). It could be possible that this association reflected some regulatory mechanism between HIC and insulin and C-peptide secretion.

4.7 Regression analysis: (Objective 5)

Although significant correlation between CST and HIC was found in black women, after adjusting for CST-HIC relationship confounders in the linear regression model, no significant association was further identified. Confounders in the analysis were all variables that produced significant correlation with HIC and among them were very strong and well known HIC determinants such as HOMA-IR, age, DIo or BMI. These, placed in the model next to CST, abolished its weaker relationship (even after removal of HOMA-IR due to collinearity). That is why two sets of models for HIC and HOMA-IR and insulin fasting are presented. The first model includes all the relevant confounders (see Chapter 3.2.2.1 for their selection) and represents the final regression output. The second model was to test whether, after the removal of certain strongly associated confounders, any significance for CST (CgA or CgA/CST ratio) could be seen.

Each of these tested predictors (CST, CgA and CgA/CST ratio) entered the models separately. For this reason, CgA, after removal of age variable from the first model, showed significance with HIC, independently of leptin and DIo. Similarly, CgA/CST ratio in a different model remained significant after removal of DIo, independently of leptin and age.

The positive association of CgA and CgA/CST with HIC is discussed above.

The final models for HOMA-IR or fasting insulin did not include any CgA or CgA/CST ratio as they were not sufficiently strongly associated to preserve their significance. The predictors of fasting insulin and HOMA-IR were the same as these two variables are strongly correlated. , these predictors were expected. If HIC is not purely

compensation for IR but is considered a primary defect (Chapter 1.2 c), (Bergman 2019), it will promote chronic hyperinsulinaemia, which eventually will proceed to insulin receptor desensitisation and target tissue IR.

Interesting is the significant relationship with adiponectin as a predictor. Adipose tissue has a connecting role between obesity and IR (Li et al., 2013). Adiponectin improves insulin sensitivity (Lihn et al., 2005) and adiponectin-KO mice showed higher IR than normal controls (Li et al., 2013). Quite recently, Ladwa et al. (2022) reported that adiponectin may be directly involved in the upregulation of HIC. Low adiponectin would be associated with low HIC and thus high insulin and HOMA-IR. Interestingly, adiponectin was not a part of the final HIC model.

Although CST and CgA/CST ratio were significantly associated with family genetic disease and/or the family monthly income, the significance was lost in the regression analysis. Inflammatory markers did not correlate with CST or CgA or their ratio.

4.8 Study limitations

1. A limitation of this study could be that the majority of volunteers were recruited from two institutes (NHLS and Wits) co-existing in one location and a convenient sampling was applied, enrolling the participants based on availability and willingness to take part. The reason for this was that the study was designed during the last stages of the COVID-19 lockdown. The safety measures had to be considered and the staff and students undergone daily facility-entry screening.

   The enrolled participants included variety of socio-economic levels (students, scientists, clinicians, technicians and technologists, nurses and cleaners) residing in different parts of Gauteng and students originating in different provinces of South Africa. However, the study population may not be fully representative of a larger population of South African black and white women and the study outcomes should be verified in a larger scale and involving insulin resistant or diabetic individuals for comparison.
2. A number of white women (43) enrolled at external enrolment points did not participate in the full OGTT and only fasting samples taken. Their fasting

glucose levels were normal and were not on any treatment impacting glucose metabolism. They were all slim and self-reported healthy, therefore, were considered to be normoglycaemic. Due to the study restricted budget, we did not have possibility to use other ways of verification such as e.g. HbA1c.

3. Only 14 white women had full OGTT completed, allowing for calculation of IGI and DIo indices. The majority of these women (11) were in NOB category. However, these results can be biased due to low numbers and should be interpreted with caution.
4. The enrolled ethnic groups were not matched for BMI. Although we have corrected for BMI in the regression analysis and designed a comparison by BMI matched groups for NOB women, the group of OB white women was missing for complete comparison. Although grouping of participants by BMI and ethnicity helped to better understand the differences related to BMI or ethnic, this was not originally intended and thus the numbers of participants in each group, especially in the OB white women group, were too low to be included into regression analysis with correction for other confounders.
5. We have not assessed participants' physical activity (all women were resting before blood collections) but white women in our study seemed to be more physically active with very healthy lifestyle. We have not included any dietary records. It is not much known on dietary effects on CST or CgA. However, the CgA proteolytic cleavage is thought to be affected by CgA hyper-glycosylation (see Chapter 1.5).
6. Throughout the study's duration, the IL-6 assay kit (E-EL-H0102) was improved by the manufacturer to a greater sensitivity (new kit: EL-H6156). This caused a problem, as the standard curve of the last purchased kit differed from the previous one. In order to have comparable results between runs, a number of samples from the previous kit were repeated in the new kit. As a consequence, only a reduced number of samples were analysed. The statistical analysis was then performed without IL-6 adjustment initially and afterwards with IL-6 adjustment due to pair-wise deletion of missing samples. Said et al. (2020) published reference intervals for IL-6 based on a large population sample. The values extended from 0 to 43.5 pg/mL with pooled estimate of 5.186 pg/mL (95% CI 4.631, 5.740). Although these values are linked to another population and include men and women, it seems that the

women in the current study had normal IL-6 results. (Detection range; 1.56-100 pg/mL).

7. The current study did not evaluate the use of contraceptives and their effects on the study outcomes.

4.9 Implications of the study:

To our best knowledge, this is the first study evaluating CST involvement in IR ethnic disparities and the first study to report significant CST (and CgA) differences between these ethnic groups. We believe that the current project findings could contribute to a wider knowledge and will stimulate further CST research and perhaps an introduction of new diagnostic tools in this field.

4.10 Follow-up research:

1. In parallel with the current study, samples for the genetic analysis were separated and stored, and the request for permission for this follow-up analysis was already included in the participants' Consent form (see **Appendix H**). We would like to look at the CST genetic variants in both ethnic populations.
2. Another follow-up study will be looking at possible effects of advanced glycation end product (AGEs) and fructose on the CgA to CST cleavage in both populations and it will include Type 2 diabetic patients as well.

Chapter 5

5. Conclusion

The research of CgA cleavage products on human health is increasing worldwide but no previous studies on glucose regulation have been carried out in South Africa. Catestatin is an endogenous peptide with beneficial pleiotropic effects, including glucose homeostasis regulation. This has been demonstrated in numerous animal-based and *in vitro* studies. Several authors have suggested a CST analogue as a novel therapeutic agent for obesity, hypertension and T2D treatment.

The current study evaluated the possible association of CST (or CgA and CgA/CST ratio) with IR disparity in normoglycaemic, normotensive and apparently healthy urban black and white South African women from the Gauteng province.

According to the current study results, only a few cases of hyperinsulinaemia and no significant ethnic difference in IR were found. However, adiposity-driven differences were observed for fasting and 30-minute insulin and HOMA-IR among black women. The DIo did not differ between groups as the IR in the OB group was compensated by significantly increased early fasting-insulin response (IGI) and reduced HIC. Although no ethnic difference for DIo or IGI was found, HIC significant ethnic differences were demonstrated and the latter supports the findings of other local and international studies. This may possibly indicate that the reduction in HIC may precede the upregulated β cell function in establishing hyperinsulinaemia.

There were no CST (CgA or CgA/CST ratio) correlations found for white women. In previous reports, CST supplements affected glucose regulation only in the presence of obesity. However, we found significantly reduced CST (and elevated CgA and CgA/CST ratio) levels in black women in comparison with white women without relationship to obesity. Although other factors not investigated in the current study may be involved, this effect could be of a genetic origin. Catestatin in black women was associated with the history of a genetic disease in the participant's family. We have not found any previous report on ethnic differences in CST circulatory concentrations.

The study has not found any direct effect of CST on IR. The study results indicate that HIC is the most closely associated variable of glucose-INS dynamics with CST (CgA and CgA/CST ratio) in the black women in our study. The association with CST was weak and lost significance when corrected for other variables in HIC predictors' model. The associations with CgA and CgA/CST ratio were weak, although, after removal of the highly associated confounders, the relationship with HIC remained significant.

Catestatin could be involved in the regulation of IR or the variables of glucose-insulin dynamics indirectly, via its effects on obesity or hypertension. The women in the study were all normotensive, normoglycaemic and apparently healthy, which might not have triggered any CST regulatory effects.

The study found a positive correlation with leptin and CST-leptin interaction has been described previously in the literature, where CST improved leptin signalling and inhibited opposing effects. The binding of CST to insulin receptor results in reduction of obesity and this, in turn, can reduce IR and improve INS and glucose levels. In the study, the OB black women had the highest leptin concentrations but relatively low CST levels (in comparison with white women). If the ethnic difference was of a genetic origin, this could indicate that relatively lower CST concentrations may be insufficient to produce obese individuals who are insulin resistant and hyperinsulinaemic.

Larger sample size with matched BMI for both ethnic groups could clarify this.

Chapter 6

6. References

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Chapter 7

7. Appendix

Appendix A

Table A1: Significant correlations of insulin fasting and metabolic and anthropometric variables with corresponding correlation coefficients and p values.

INS F		**Black Women (n=66)**	**White Women (n=52)**
Age &	Age (-0.220; 0.017)	GLC-F (0.477; <0.001)	C-PEP (0.849; <0.001)
Metabolic	GLC-F (0.268; 0.003)	C-PEP (0.885; <0.001)	HOMA-IR (0.991; <0.001)
Variables:	C-PEP (0.824; <0.001)	GLC-30' (0.471; <0.001)	HIC (-0.825; <0.001)
	HOMA-IR (0.992; <0.001)	INS-30' (0.439; <0.001)	Leptin (0.448; 0.001)
	CgA (-0.261; 0.004)	HOMA-IR (0.993; <0.001)	IL-6 (0.456; 0.001)
	HIC (-0.831; <0.001)	**CgA (-0.401; 0.001)**	
	Leptin (0.522; <0.001)	**CgA/CST (-0.418; <0.001)**	
	Adipon. (-0.361; <0.001)	HIC (-0.825; <0.001)	
	IL-6 (0.349; <0.001)	DIo (-0.470; <0.001)	
		Leptin (0.475; <0.001)	
		Adipon. (-0.281; 0.022)	
Anthropo-	BMI (0.528; <0.001)	BMI (0.510; <0.001)	BMI (0.434; 0.001)
metric	WC (0.499; <0.001)	WC (0.397; 0.001)	WC (0.495; <0.001)
Variables:	HC (0.473; <0.001)	HC (0.369; 0.002)	HC (0.448; 0.001)
	WHtR (0.482; <0.001)	WHtR (0.350; 0.004)	WHtR (0.494; <0.001)
	WHR (0.292; 0.001)	WHR (0.244; 0.049)	

GLC-F: fasting glucose, C-PEP: C-peptide, GLC-30': 30-minute glucose, INS-30': 30-minute insulin, HOMA-IR: homeostatic model assessment for insulin resistance, DIo: disposition index, Adipon.: adiponectin, HIC: hepatic insulin clearance, CgA: Chromogranin A, CgA/CST: Chromogranin A to Catestatin ratio, IL-6: interleukin-6, WHR: waist to hip ratio, WC: waist circumference, HC: hip circumference, WHtR: waist to height ratio.

Appendix B

Table B1: Significant correlations between HOMA-IR and metabolic and anthropometric variables with corresponding correlation coefficients and pvalues.

HOMA-IR	All Participants (n=118)	Black Women (n=66)	White Women (n=52)
Metabolic Variables:	GLC-F (0.390; <0.001)	GLC-F (0.577; <0.001)	GLC-F (0.328; 0.018)
	INS-F (0.992; <0.001)	INS-F (0.993; <0.001)	INS-F (0.991; <0.001)
	C-PEP (0.837; <0.001)	GLC -30' (0.571; <0.001)	C-PEP (0.843; <0.001)
	CgA (-0.266; 0.004)	INS- 30' (0.428; <0.001)	HIC (-0.815; <0.001)
	HIC (-0.804; <0.001)	C-PEP (0.889; <0.001)	Leptin (0.460; 0.001)
	Leptin (0.518; <0.001)	**CgA (-0.404; 0.001)**	IL-6 (0.438; 0.002)[#2]
	Adipon (-0.337; <0.001)	**CgA/CST (-0.408; 0.001)**	
	IL-6 (0.324; 0.001)[#1]	HIC (-0.804; <0.001)	
		DIo (-0.476; <0.001)	
		Leptin (0.478; <0.001)	
		Adipon. (-0.250; 0.043)	
Anthropo-metric Variables:	BMI (0.523; <0.001)	BMI (0.516; <0.001)	BMI (0.428; 0.002)
	WC (0.487; <0.001)	WC (0.390; 0.001)	WC (0.507; <0.001)
	HC (0.466; <0.001)	HC (0.378; 0.002)	HC (0.453; 0.001)
	WHtR (0.468; <0.001)	WHtR (0.341; 0.005)	WHtR (0.515; <0.001)
	WHR (0.279; 0.002)		WHR (0.284; 0 .042)

[#1]n=100; [#2]n=46; GLC-F: fasting glucose, INS-F: fasting insulin, C-PEP: C-peptide, GLC-30': 30 minute glucose-, INS-30': 30-minute insulin-, BMI: body mass index, HOMA-IR: homeostatic model assessment for insulin resistance, WC: waist circumference, HC: hip circumference, HIC: hepatic insulin clearance, Adipon.: adiponectin, DIo: disposition index, WHR: waist to hip ratio, WHtR: waist to height ratio, CgA: chromogranin a, IL-6: interleukin-6, CgA/CST: chromogranin a to catestatin ratio.

Appendix C

Table C1: Significant correlations between HIC and metabolic and anthropometric variables with corresponding correlation coefficients and p values.

HIC	All participants (n=118)	Black Women (n=66)	White Women (n=52)
Age & Metabolic Variables:	Age (0.365; <0.001)	Age (0.245; 0.047)	Age (0.309; 0.026)
	INS-F (-0.831; <0.001)	**CST (-0.247; 0.045)**	INS-F (-0.825; <0.001)
	C-PEP (-0.369; <0.001)	**CgA (0.366; 0.002)**	C-PEP (-0.401;0.003)
	HOMA-IR (-0.804; <0.001)	**CgA/CST (0.387; 0.001)**	HOMA-IR (-0.815; <0.001)
	Leptin (-0.484; <0.001)	GLC-F (-0.277; 0.024)	Leptin (-0.356;0.010)
	Adipon. (0.421; <0.001)	INS-F (-0.825; <0.001)	Adipon. (0.321;0.020)
	MCP-1 (-0.210; 0.023)	GLC-30' (-0.272; 0.027)	IL-6 (-0.365; 0.013)[#2]
	IL-6 (-0.420; <0.001)[#1]	INS- 30' (-0.374; 0.002)	
		C-PEP (-0.462; <0.001)	
		HOMA-IR (-0.804; <0.001)	
		DIo (0.423; <0.001)	
		Leptin (-0.3724;0.002)	
Anthropo-metric Variables:			
	BMI (-0.441; <0.001)	BMI (-0.377; 0.002)	BMI (-0.318;0.021)
	WC (-0.422; <0.001)	WC (-0.251; 0.042)	WC (-0.326;0.018)
	HC (-0.431; <0.001)	HC (-0.246; 0.047)	HC (-0.334;0.015)
	WHtR (-0.448; <0.001)		WHtR (-0.363;0.008)
	WHR (-0.206; 0.025)		

[#1]n=100; [#2]n=46

GLC-F: fasting glucose, INS-F: fasting insulin, C-PEP: C-peptide, GLC-30': 30- minute glucose-, INS-30': 30-minute insulin-, BMI: body mass index, HOMA-IR: homeostatic model assessment for insulin resistance, WC: waist circumference, HC: hip circumference, HIC: hepatic insulin clearance, Adipon.: adiponectin, DIo: disposition index, WHR: waist to hip ratio, WHtR: waist to height ratio, CgA: chromogranin a, IL-6: interleukin-6, CgA/CST: chromogranin a to catestatin ratio.

Appendix D

Table D1: Significant correlations between HIC and metabolic and anthropometric variables with corresponding Regression Coefficient R and p-values.

IGI	**Black women (n=66)**
Metabolic Variables:	INS-30' (0.473; <0.001) DIo (0.796; <0.001) Leptin (0.290; 0.018)
Anthropometric Variables:	BMI (0.328; 0.007) HC (0.250; 0.043) ABSI (-0.318; 0.009)
DIo	**Black women (n=66)**
Metabolic Variables:	GLC-F (-0.299; 0.015) INS-F (-0.470; <0.001) GLC-30' (-0.295; 0.016) C-PEP (-0.384; 0.001) HOMA-IR (-0.476; <0.001) HIC (0.423; <0.001) IGI (0.796; <0.001)
Anthropometric Variables:	No significant correlations

GLC-F: fasting glucose, GLC-30': 30-minute glucose-, INS-F: fasting insulin, INS-30': 30- minute insulin, C-PEP: C-peptide, BMI: body mass index, DIo: disposition index, HC: hip circumference, ABSI: body shape index, IGI: insulinogenic index, HIC: hepatic insulin clearance.

Appendix E: Study Invitation Letter

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

NATIONAL HEALTH LABORATORY SERVICE

INVITATION LETTER
Research Study

Association of Catestatin with Insulin Resistance Disparity in Normoglycaemic Black and White South African Women

IF YOU ARE A WOMAN WITH AFRICAN-ANCESTRY OR CAUCASIAN, 18-50 YEARS OLD, WITHOUT ANY CHRONIC METABOLIC DISEASE,

PLEASE, COME TO HELP US with donating a few blood samples!!!

Good day,

We are scientists from the Department of Chemical Pathology at the NHLS-Wits Medical School conducting a study on black vs white women disparities in glucose metabolism. For still unknown reason, there are worldwide-existing ethnic disparities in glucose metabolism. They manifest in higher insulin resistance among women of African-descent when compared with their White counterparts. Insulin resistance is a risk factor for the development of Type 2 diabetes.

Among factors implicated in insulin resistance are obesity, inflammation and hereditary factors. We would like to investigate, whether low levels of peptide catestatin could not be another contributing factor. This body-natural product, in a beneficial manner, is involved in the regulation of glucose metabolism. It reduces obesity and inflammation. Catestatin analogues were already suggested for the treatment of obesity and hypertension.

If you want to know details of the study and come to help us, please contact:

Ms PHILISIWE NTULI (Principal Investigator)
Wits Medical School, **Room 3Q07**
Department of Chemical Pathology
Telephone number: 011 489 8448
e-mail: philisiwe.ntuli@nhls.ac.za

Alternatively: Study supervisors:
Dr Marketa Toman; 082 394 5817
marketa.toman@nhls.ac.za
Ms Tracy Snyman; 011 489 8582
tracy.snyman@nhls.ac.za

The study has been approved by the Wits medical Human Research Ethics Committee: (HREC) under the number: Ref: 14/49 M200922; 19.1.2021)
HREC-Medical.ResearchOffice@wits.ac.za

Appendix F

Association of Catestatin with Insulin Resistance Disparity in Normoglycaemic Black and White South African Women

Study Inclusion Questionnaire

1. General Information

Interview No: _____

Participant: **Study number** ☐

Name & Surname _____

Date of Birth _____

Department/Ward/Student's number _____

Contact phone number/e-mail _____

_____

Ethnic origin: Black SA _____White SA _____

2. Medical History:

Are you NOT: pregnant or breast-feeding, menopausal or postmenopausal?

Have you been diagnosed/ currently treated for any of the following conditions/ diseases? (Mark below only yes/no, you do not have to specify. If you have any question, please ask)

- Diabetes or abnormal blood glucose tests
- Cardiovascular disease; chronic heart failure;
- High cholesterol; atherosclerosis
- Hypertension
- Kidney disease/dysfunction
- Any cancer, anemia or any other blood disorder
- Current treatment with anticoagulants
- Inflammatory disease (e.g. rheumatoid arthritis, systemic lupus erythematosus (SLE), inflammatory bowel disease)
- Current treatment for heartburn/ ulcers (Omeprazole/ Trustan/ Nexium…)
- Current treatment for Allergies (antihistamines)
- Current treatment with steroid hormones (cortisone)
- HIV or TB
- Thyroid disorder; chronic obstructive pulmonary disease (COPD)

☐YES ☐NO

Do you have any other chronic disease? ☐Yes ☐No

Any known genetic disease in the family? ☐Yes ☐No

Do you smoke? ☐Yes ☐No

Do you drink alcoholic beverages? ☐Yes ☐No

If yes, how often? ☐Occasionally ☐Often/_____per day/ per week

Comments: _____

---------- Date:----------

3. Anthropometry and BP:

Weight:_____[kg]; Height: _____[m]; BMI: _____kg/m²;

Hip circumference_____[cm]; Waist circumference_____[cm]

Blood pressure: _____[mmHg]

Fasting Glucose (by glucometer)---------- mmol/L

Glucose level at 120 min of OGTT (finger prick): _____[mmol/L]

NOTES:_____

_____

4. Outcome:

Enrolled: ☐Yes ☐No

Signature (researcher): _____

Date: _____

Appendix G

Study Socio-Economic Status questionnaire

Association of Catestatin with Insulin Resistance Disparity in Normoglycaemic Black and White South African Women

ASSESSMENT OF SOCI-ECONOMIC STATUS QUESTIONNAIRE

Please circle the appropriate selection

A. Level of Education

1) Illiterate N/A

2) Primary school level (<12 years of age)

3) Standard 6 – 7 (12-14 years of age)

4) Standard 8 – 12, (matriculation; 14-18 years of age)

5) Post matriculation, tertiary education (>18 years of age), Diploma

B. Family monthly income categories:

The scale below is based on salary ranges from R 4,249 (minimum wage) to R 139,013 (maximum wage) per month in the pay scale South Africa, with an average of R 30, 765. https://briefly.co.za/30142-what-average-salary-south-africa-2019.html

1) Below or equal to R 10 000

2) Over R 10 000 but below or equal to R 20 000

3) Over R 20 000 but below or equal to R 40 000

4) Over R 40 000 but below or equal to R 60 000

5) Over R 60 000

- Number of adults in the household _____
- Number of children _____

Appendix H

Study informed consent

INFORMED CONSENT
Research Study
Association of Catestatin with Insulin Resistance Disparity in Normoglycaemic Black and White South African Women

STUDY INFORMATION

Good day,

Thank you for responding to our invitation letter and showing interest to know more about our study.
I am Philisiwe Ntuli, working for the National Health Laboratory Services and this research is part of my Master's degree that I am enrolled in at the University of the Witwatersrand. We are doing research on the possible involvement of the peptide Catestatin in insulin resistance (IR) disparities between South African Black and Caucasian women residing in the Gauteng province.

What is this study about?

Type 2 diabetes is a serious health problem, associated with high morbidity, which incurs large costs to society. The poorer glucose control (higher insulin resistance) in African-descent women represents a greater risk for the development of Type 2 diabetes. Most people with Type 2 diabetes are overweight or obese and obesity is a strong component in the aetiology of insulin resistance. However, IR is a complex metabolic disorder with multiple etiological pathways and its exact cause is still not well understood.

Catestatin, the target of our research, is a peptide occurring in the body, which is derived from the cleavage of glycoprotein Chromogranin A in pancreatic B cells. These cells are responsible for the production and secretion of the hormone insulin, which regulates the glucose in the body. High insulin blood levels indicate IR, which is the inability of insulin to control well the blood glucose.

From animal studies, it is known that Catestatin acts in a similar way to insulin and helps in glucose regulation. In addition, it reduces obesity and acts as anti-inflammatory. These are all opposite features to those associated with insulin resistance.
We think that African Black women have low levels of Catestatin, which could result in the impairment of the above regulations. If this is true, a novel treatment in a form of Catestatin supplements may reduce obesity, inflammation, and the risk of progression to Type 2 diabetes in these women. Our research can add to this knowledge.
What is involved in the study participation?

This study includes South African Black and White women 18 to 50 years old, non-diabetics, apparently healthy (no chronic metabolic disease/ treatment) with normal glucose levels and blood pressure and BMI between 18-46 kg/m^2, non- pregnant or breast feeding.

Study diagram:

A. Inclusion Questionnaire → Not selected
(5-15 minutes; Chem. Path.)

↓ **Pre-selected**

B. Anthropometrical and → Not enrolled
Blood pressure measurements
(15 minutes; Day ward/Chem Path)

↓ **Enrolled**

C. OGTT at F and 30-minutes,
2-hour glucose by finger-prick → Excluded
(40 minutes and coming back
For 5 minutes exactly 2hours after
the glucose drink; Day ward);
SES Questionnaire between blood collections

↓ **End of the study** → After finishing the sample analysis, participants will obtain their results

A. To include required participants for this study, we need to ask you about and evaluate if you have any metabolic diseases, conditions or treatments that may bias the study outcomes. This will help to ensure that you are fulfilling the study inclusion criteria. This means that you can still be excluded from the study. We will be required to take your contact numbers and personal details. The above-mentioned factors will be used to ensure that the outcome is due to the Catestatin effect and not due to any other causes.

The information in this Questionnaire and all other study-related information and results **will always be kept confidential** and will only be accessible to the research team. If you will not be enrolled into the study, we will return/destroy the questionnaire. If you will be selected, your file as well as blood samples will be **coded by a number (anonymised)** so your data will be handled as confidential until destroyed. The principal researcher will decode the data only for reporting your blood results to you (or GP). The report will be given directly to you. **The data identity will always be protected from unauthorised access** (records will be kept under the lock and key and computer data will be password protected).
The overall study findings will be presented in conferences and/or research journals.

B. If pre-selected into the study, we will ask you to come to the Chemical Pathology Day ward in the Green Block, Area 454, Room 1, adjacent to the Main Street level 5 in the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) between 7.30am and 8.00am (this can be arranged as you need) after overnight fasting. This means that you can have your last meal or drink at 22.00 hours the night before the visit. You must not have anything to eat or drink or any sweet, chewing gum and must not smoke the morning of the visit. First, we will take your weight, height, waist and hip circumference and blood pressure. From the measurements, we will calculate body mass index (BMI). We will enroll all women with a BMI between 18 kg/m^2 and 35 kg/m^2 who are non-hypertensive. If your BMI is less than or greater than this, or your blood pressure is high, you will be excluded from this study. If you were enrolled, the measurements will be used to correct results for as described in the step A. If requested by you, we can separate the measurements and the OGTT into two visits.

C. If you were enrolled and came fasting, we will proceed with the OGTT, which is a routine test to detect your glycaemia (sugar levels), including Type 2 diabetes.

OGTT procedure: The nurses in the Day ward will ask you to sit down, a small needle will be inserted into a vein in your arm and four-five small tubes (20 ml; it is like 4 teaspoons) of blood sample will be taken. This is accompanied by a small discomfort but no pain or risk to you.
Subsequently, you will be asked to drink 75g of glucose (sugar) dissolved in water. You will stay seated, and after 30 minutes, another blood collection of 5 ml (2 halfway full tubes only; approximately 1 teaspoon).
Between these two blood collections, you will help us to fill in a short **Questionnaire** on your Socio-Economic Status (**SES;** such as education/ income range…), as these are additional variables, the study needs to correct for.
In a standard OGTT test, the blood is collected further at 1 hour and 2 hours but for our study, we need only the first two values. However, to detect how your glycaemia is controlled after drinking glucose, we need to look at the 2-hour results. We only can enroll women with normal glucose levels. Therefore, you will be asked to come back in 1h and 15 minutes to finish the test. We will not be drawing blood anymore; we will test your glucose levels by a finger-prick and a portable glucose-meter.
Once this is complete, your participation is finalised. However, if your glucose levels indicate an abnormality in glucose metabolism, we will not be able to include you in the study. Nevertheless, you will be given your results and a note for follow up at your GP.

The collected blood samples will be analysed for following: glucose; insulin and C-peptide – hormones regulating glucose metabolism; Chromogranin A and catestatin – our primary target which may be involved in this regulation; leptin and adiponectin – related to obesity; Interluekin-6 (IL-6) and Monocyte chemoattractant protein-1 (MCP-1) – associated with inflammation.

If you have any questions or do not understand anything fully, please ask.
You do not have to decide today whether you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with, about the research.

If you wish to participate, please go to the **next two pages**, and read carefully. If you understand and agree to participate in this research study, please sign the Consent Form.

Association of Catestatin with Insulin Resistance Disparity in Normoglycaemic Black and White South African Women

CONSENT FORM to participate in this research study.

There is no direct benefit to the participant from the outcome of this study. The ***potential benefits*** of taking part in this study are that you will be informed about the status of your glucose metabolism (if you are healthy, or if it is impaired or even may be a diabetic and do not know about it). You will receive your results from the other tests with explanation. If during our study, any results from the tests show abnormal levels, you will be consulted and a note for your GP will be handed to you. There are **no known risks or side effects** for you by participating in this study, except for a slight discomfort during the insertion of needle or finger-prick for the blood collection. There is **no reimbursement** in this study.

All volunteers that participate in the study will contribute to the knowledge, which in the future may lead to a novel treatment, reducing the risk or even preventing the progression to Type 2 diabetes.

Participation in this study is voluntary and you are free to refuse to participate or withdraw your consent and to discontinue participation at any time. Such refusal or discontinuance will not be taken against you in any way. A signed copy of this consent form will be made available to you.

I have been fully informed as to the procedures to be followed, the purpose, possible risks, and benefits of the study.
In signing this consent form, I agree to participate in this study and undergo the above-mentioned procedures. I understand my right to refuse to participate or withdraw my consent in this study at any time.
I understand that if I have any related questions at any time, they will be answered.

Date: _____ Participant's name: _____Signature:_____

I have fully informed the participant, explained, and answered all questions asked.

Date: _____ Researcher's name: _____Signature: _____

Contact numbers:
Principal Investigator: Ms. Philisiwe Ntuli; 011 489 8448; philisiwe.ntuli@nhls.ac.za
Main supervisor: Dr M. Toman; 082 3945817; marketa.toman@nhls.ac.za
Co-supervisor: Mrs. Tracy Snyman; 011 489 8582; tracy.snyman@nhls.ac.za
The Day ward in the Depart. of Chemical Pathology, TEL: 011 489 8503

This study has been reviewed and approved by the Wits University medical Human Research Ethics Committee (HREC) under the number: Ref: 14/49 M200922; 19.1.2021
HREC contact numbers:
HREC-Medical.ResearchOffice@wits.ac.za;
Research Administrator – Mr Rhulani Mkansi Rhulani.Mkansi@wits.ac.za or Ms Zanele Ndlovu zanele.ndlovu@wits.ac.za – 011 717 1252/2700/1234/2656

Association of Catestatin with Insulin Resistance Disparity in Normoglycaemic Black and White South African Women

CONSENT FORM

To retain the collected samples and information for the period of 5 years at maximum from completion of this study
For the purpose of an additional research.

Dear participant,

The research team would like to store the remnant samples after completing this study for following reasons:

- In the case, this study proves the hypothesis that protein catestatin may regulate glucose metabolism in a different manner in Black women than in White women, this will need to be investigated further. It can result in a valuable information and possible suggestion for a treatment to prevent progression to Type 2 diabetes in Black women.
- If the study will not find the expected outcome, as researchers we learn from the results and may want to try a different approach.

But for both cases, this can only be done on the same samples as in the original study. For this purpose, we request your permission to store the samples for future Chromogranin A- related studies. The samples will be kept in -75^{o}C freezer in the Department of Chemical Pathology, NHLS-Wits Medical School building, level 3. The samples will remain coded with the study numbers. The data from questionnaires and measurements will remain coded. The decoding list will be password protected in computer. Access will be available to the research team only. Any hard copy link will be destroyed after completion of the original study. Any new research project using these samples and/or data will submit a new application to the Wits Ethics Committee (medical; HREC) and will request permission from at least one member of the current research team (of 3 scientists).

In signing this consent form, I agree to following: (Click the appropriate selection)

- **My coded samples and coded data may be stored for another 5 years after completion of the current project for the purpose of a new but related study.**
- **My coded samples and coded data may be stored as above but I want my name link to be removed from the electronic storage.**
- **I wish my samples/data to be destroyed right after completing the current study.**

I understand my right to change/withdraw this consent at any time, which will not be taken against me in any way. A signed copy of this consent form will be made available to me.
I understand that if I have any related questions at any time, they will be answered.

Date:_____ Participant's name: _____Signature:_____

I have fully informed the participant, explained, and answered all questions asked.

Date:_____ Researcher's name: _____Signature: _____

Appendix I: Study poster

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG
FACULTY OF HEALTH SCIENCES
NATIONAL HEALTH LABORATORY SERVICE

PARTICIPANTS NEEDED!!

We are Researchers from the Chemical Pathology Department at Wits medical school, we are conducting a study investigating glucose regulation disparities between Black and White South African Women.

We are still looking for White women volunteers to donate a fasting blood sample.



Who can participate?

White South African Women between the age of 18 and 50 years without any known chronic metabolic disease or hormonal treatment.

Participation involves:

- Enrolment questionnaires
- Anthropometric data and blood pressure measurement.
- Overnight fasting blood sampling.

Data made available to you includes:

- Glucose, insulin, C-peptide, chromogranin A, catestatin, adiposity & inflammatory markers.

Please take this opportunity and join the study!

For more information:

Please contact: 082 500 2971
Philisiwe Ntuli (Principal Investigator)
Email: philintuli@yahoo.com / philisiwe.ntuli@nhls.ac.za

Location:

Wits Medical School, Room 3Q07
Department of Chemical Pathology
Tel: 011 489 8448

Study approved by the Wits Human Research Ethics Committee (M200422)

Appendix J: Ethics certificates

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Postgraduate Affairs

TO:	Mlles P Ntuli and T Snyman School of Pathology Department of Chemical Pathology Medical School E-mail: philintuli@yahoo.com
CC:	Supervisor: Dr M Toman <Marketa.Toman@nhls.ac.za> and <HREC-Medical Research Office@wits.ac.za>
FROM:	Mr Iain Burns Human Research Ethics Committee (Medical) Tel: 011 717 1252 E-mail: Iain.Burns@wits.ac.za
DATE:	2021/01/19
REF:	R14/49
PROTOCOL NO:	**M200922** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)
PROJECT TITLE:	Association of catestatin with insulin resistance disparity in normoglycaemic Black and White South African women

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.



MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE
WITWATERSRAND
JOHANNESBURG

R49 Mlles P Ntuli and T Snyman

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200922

NAME:
(Principal Investigator) Mlles P Ntuli and T Snyman

DEPARTMENT: School of Pathology
Department of Chemical Pathology
Medical School
University

PROJECT TITLE: *Association of catestatin with insulin resistance disparity in normoglycaemic Black and White South African women*

DATE CONSIDERED: 2020/10/02

DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Dr M Toman

APPROVED BY: Dr CB Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL: 2021/01/19

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **September** and therefore reports and re-certification will be due in the month of **September** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

22/01/2021
Date

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2023/08/28

Mlles P Ntuli and T Snyman
School of Pathology
Department of Chemical Pathology
Medical School
University

Sent by e-mail to: philintuli@yahoo.com

Dear Ms Ntuli

Re: **Protocol Ref No:** M200922
Protocol Title: *Association of catestatin with insulin resistance disparity in normoglycaemic Black and White South African women*
Principal Investigator: Mlles P Ntuli and T Snyman

Thank you for your letter of 2023/08/16.

We note and approve of your proposal to attract more White female participants by:

1. sampling at fasting only
2. raising the upper age limit for participants from 45 to 55 years
3. adding Wits Donald Gordon Medical Centre as a recruitment site, subject to permission from the CEO

Thank you for keeping us informed.

Yours Sincerely

I. Burns

.....

Mr I Burns
For the Human Research Ethics Committee (Medical)

.....

Professor P Ruff, Chairperson, Human Research Ethics Committee (Medical)