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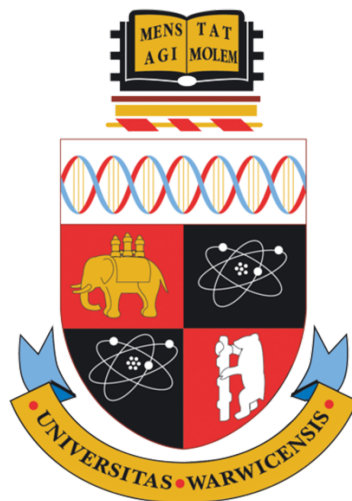
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The Influence of Vitamin D on Metabolic Disease and Adipose Tissue Metabolism

by

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for the degree of

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ABBREVIATIONS

%	Percent
Δ Ct	Delta threshold cycle
1,25(OH) ₂ D ₃	Calcitriol, active vitamin D ₃ , or 1-alpha-25-Dihydroxy-cholecalciferol
25(OH) ₂ D ₃	Calcidiol, circulating vitamin D, 25-hydroxycholecalciferol, or 25-hydroxyvitamin D
20x	20 times magnification
40x	40 times magnification
Abd Sc	Abdominal Subcutaneous adipose tissue
AKT	Akt, also known as protein kinase B (PKB)
AT	Adipose tissue
BMI	Body mass index
BPD	Bilio-pancreatic diversion
BS	Bariatric Surgery
CD14	Cluster of differentiation 14
CD68	Cluster of differentiation 68
cDNA	Complimentary deoxyribonucleic acid
Chol	Cholesterol
CYP27A1	Cytochrome P450 Family 27 Subfamily A Member 1
CYP2J2	Cytochrome P450 Family 2 Subfamily J Member 2
CYP2R1	Cytochrome P450 Family 2 Subfamily R Member 1
DMEM/F12	Dulbecco's modified eagle medium/nutrient mixture F-12
ECL+	Enhanced chemiluminescence plus
EDTA	Ethylenediaminetetraacetic acid (EDTA)
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
EWL	Excess weight loss
GLUT4	Glucose transporter 4
HbA1c	Glycosylated haemoglobin
HDL	High-density lipoprotein
HOMAIR	Homeostatic assessment model of insulin resistance
IL-1 β	Interleukin - 1 beta
IL-6	Interleukin - 6
IL-8	Interleukin - 8
IR	Insulin receptor
IRS1	Insulin receptor substrate 1
JNK	c-Jun N terminal kinase
kDa	Kilodalton
LAGB	Laparoscopic adjustable gastric banding
LDL	Low-density lipoprotein
LSG	Laparoscopic Sleeve Gastrectomy

MCP1	Monocyte Chemoattractant Protein-1
Na ₃ VO ₄	Sodium vanadate
NaF	Sodium fluoride
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
Om AT	Omental adipose tissue
PBS	Phosphate buffered saline
qRT-PCR	Quantitative real-time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
SG	Sleeve gastrectomy
SGBS	Simpson–Golabi–Behmel syndrome
T2DM	Type 2 Diabetes Mellitus
T75	75 square centimetre growth area tissue culture flask
TG	Triglyceride
TLR	Toll like receptor
TNF-alpha	Tumor necrosis factor alpha
VDR	Vitamin D receptor
VitD	Vitamin D
WAT	White adipose tissue
WHR	Waist hip ratio
μCi/mL	Microcurie per millilitre

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DECLARATION

All sentences or passages quoted in this project dissertation from other people's work have been specifically acknowledged by clear cross referencing to author, work and page(s). I understand that failure to do this amounts to plagiarism and will be considered grounds for failure in this module and the degree examination as a whole.

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (included data generated and data analysis) was carried out by the author, except in the cases outlined below:

All patient data outlined in Chapter 3 was collected between 2011 and 2018 by staff at UHCW Specialist Weight Management Service, in particular Neha Shah (Bariatric Dietitian), Jenny Abraham (Bariatric Nurse), Dr. Milan Piya (Consultant Endocrinologist) and Mr. Vinod Menon (Consultant Surgeon). A full list of staff who participated in the care and data gathering of patients during their treatment is acknowledged by the author and may be found in Appendix 1.

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ABSTRACT

Obesity is characterised as a chronic inflammatory state in which adipose tissue acts as a mediator of metabolic dysfunction leading to type 2 Diabetes Mellitus (T2DM). Several factors may influence this inflammatory state, with a considered influence due to arise through altered circulating levels of vitamin D (25(OH)D) which is known to be a common nutrient deficiency in obesity. Several studies to date have implicated low vitamin D (VitD) levels in association with the increased insulin resistance and T2DM, though the role of adipose tissue and its metabolism within adipose tissue remains unclear. As such, this thesis sought to investigate, (1) baseline VitD levels as a potential predictor of beneficial metabolic outcomes in participants undergoing bariatric surgery; (2) the *in vitro* association of VitD in different metabolic states and its influence on human adipocyte metabolism; (3) the influence of adiposity on VitD levels and metabolism. These investigations revealed several findings, specifically that (1) baseline VitD predicted 27% ($p < 0.001$) of the presence of T2DM post-bariatric surgery and predicted 10% of weight regained at 18 months post-operatively ($p = 0.029$); (2) the *in vitro* treatment with 10nM and 100nM VitD significantly downregulated inflammatory markers ($P < 0.05$) and improved/restored glucose uptake in insulin resistant SGBS human adipocytes (21-37% rise respectively; $P < 0.05$) through inhibition of mitogen-activated protein kinase (MAPK) and NF κ B pathways and stimulating GLUT4 translocation; and (3) VitD metabolic components (metabolizing enzymes and VDR) within AT are affected by adiposity and their expression levels are inversely associated with circulating VitD ($p < 0.05$). In summary, these findings identify that VitD appears to reduce inflammation and restores glucose uptake in SGBS human adipocytes, and highlights the importance of VitD levels as an independent indicator for successful T2DM remission and weight loss, 12 months following sleeve gastrectomy (SG). Therefore, the use of baseline VitD levels may help us predict those more likely to achieve better health outcomes, as well as prioritize post-surgery support to achieve success for all participant groups. In addition, since VitD levels are associated with AT content of VitD hydroxylases, that are associated with body weight and fat mass, individuals with greater weight or fat mass may require higher doses of VitD to reach acceptable circulating levels.

CHAPTER 1
Introduction and Aims

1.1 Obesity, Adipose Tissue and Metabolic Disease

1.1.1 Definition of Overweight and Obesity

In clinical practice, body fat is estimated by a simple and frequently used formula that compares weight to height, the body mass index (BMI Kg/m²). It is the most frequently used method in clinical and epidemiological studies which gives an indication that variation in weight for a group of the same height is due to fat mass. Several practical methods are used to assess body fatness including BMI, waist circumference, skinfold thickness, bioelectrical impedance analysis and dual-energy X-ray absorptiometry (DXA) [1, 2]. Each method has its advantages and limitations (Table 1.1.1), however, a graded classification of overweight and obesity with valuable information about increasing body fatness can be obtained by BMI. Using BMI allows meaningful assessments and comparisons of weight status within and between population groups and the identification of persons who are at risk of morbidity and/or mortality. It also allows identification of priorities for treatment options at an individual or community level and for assessing the effectiveness of interventions such as bariatric surgery. It is important to note that BMI may not reflect the actual level of adiposity across different groups nor does it comprise the broad variations in the nature of obesity between different individuals and populations [1, 3], however, for comparison of body fatness, BMI seems a useful measure independent to age and sex [4]. An expert committee from World Health Organization (WHO) has proposed the classification of overweight and obesity that applies to both gender and to all adult age groups (Table 1.1.2) [5, 6].

Method	Definition	Advantages/disadvantages
Body mass index (BMI)	Body mass in kilograms divided by square of the body height in meters	BMI is strongly associated with densitometry measurements of body fat mass; main drawback is that it does not discriminate fat mass from lean mass
Waist circumference (WC)	The circumference of the abdomen in centimetres in between lower border of ribs and top of the hip bone	WC and waist-to-hip ratio provide measures for assessing body fat distribution; however, they do not provide precise estimates of intra-abdominal (visceral) fat
Skinfold thickness	Measurement of the thickness of a “pinch” of a skin (in centimetres) using callipers provides a more precise assessment if taken at various sites	Measurements are subject to substantial differences between observers, require precise callipers and do not provide any data on intramuscular and abdominal fat
Bio-impedance	This equipment sends a safe, small and imperceptible, electric current through the body, to measure the resistance. The current faces less resistance passing through lean body than it does passing through body fat mass and water. Equations are used to estimate fat-free mass and body fat percentage	Simple and practical devices but neither measures fat nor predict biological outcomes more precisely than simpler anthropometric measurements
Dual-energy X-ray absorptiometry (DXA)	The DXA system measures bone mineral density (BMD) and soft tissue by two -ray beams. BMD can be determined by subtracting soft tissue absorption.	Been considered the ‘gold standard’ for estimating body fatness because of its accuracy and ease of measurement for both the patient and the technician. However, it has been limited to the research setting because of their cost and complexity.

The definition of a healthy weight for each specific group, for example for elderly, presents challenges. First, the definition of healthy weight is always based on total death rates which can be ambiguous. For instance, some people lose weight as a consequence of a fatal disease which might be unrecognized at the time of the survey, but results in mortality. This implies higher death rates among those with lower weights and is called as reverse causation. Second, the presence

of confounding factors is considered to be a major concern that might interfere with the association between body weight and mortality [1]. In a 17 years prospective study, 116,000 US women were studied and the results showed a U-shaped association between BMI and mortality rates after adjustment for most confounders. However, when the analysis was limited to non-smokers and the reverse causation was accounted for, the relationship became a simple direct association [7]. Despite these drawbacks in BMI calculation, there is a strong correlation between BMI and the incidence of several chronic diseases caused by excess fat, including type 2 diabetes (T2DM), insulin resistance (IR), hypertension, cardiovascular diseases and cholelithiasis. This association is approximately linear for a range of BMI indexes below 30 Kg/m², but all risks are significantly increased for those subjects with a BMI above 29, for male and female [8-12]. In addition to BMI, waist circumference (WC) shows a positive relationship with several metabolic diseases such as chronic heart disease, hypertension and blood lipids profile. The choice of cut-off points on the WC range involves a trade-off between specificity and sensitivity similar to that for BMI. The cut-off points for WC are also gender-specific and may be of guidance to predict risk of metabolic complications (Table 1.1.3) [13]. A WC of level 1 is used to alert health care practitioners to potential risk, whereas level 2 should initiate therapeutic intervention [14, 15].

Table 1.1.2 Proposed cut-off points by a WHO expert committee for the classification of body weight

BMI (kg/m ²)	WHO classification	Popular description
<18.5	Underweight	Thin or Slim
18.5 – 24.9	Normal	‘normal’, ‘healthy’, ‘acceptable’
25.0 – 29.9	Pre-obesity	Overweight
30.0 – 34.9	Obesity class I	Obesity
35.0 – 39.9	Obesity class II	Obesity
>40	Obesity class III	Morbid obesity

Table 1.1.3 Waist circumference cut-off points and prediction of metabolic complications

	Increased risk	Substantially increased risk
Male	≥ 94 cm	≥ 102 cm
Female	80 - 88 cm	≥ 88

Gender-specific waist circumferences (WC) are displayed that indicate (level 1) as ‘increased risk’ and (level 2) as ‘substantially increased risk’ of obesity associated metabolic complications. Level 1 is intended to alert health care providers to potential risk for chronic heart diseases whereas level 2 should initiate therapeutic interventions.

1.1.2 Obesity pandemic and economic burden

Obesity can be defined as a disease characterized by excess body fat accumulation leading to adverse health effects. In the UK, it is estimated that the total extra costs to the UK National Health Service (NHS) from having an overweight women (with a BMI of 25–29.9 kg/m²) were 30% of the total additional budgets from all the women with a BMI of 25 Kg/m² or above, which represents a substantial expenditure of health-care costs [16].

In addition to economic burden, obesity constitutes an essential threat to global public health. Therefore, it is important to estimate the prevalence in order to identify those at risk and establish resources to combat obesity and its consequences worldwide. Obesity as defined as a BMI above 30 kg/m² and is prevalent in all continents. Globally, the prevalence of overweight and obesity

have almost tripled since 1972 reaching 39% and 19.5% among adult population in 2016 respectively (Figure 1.1.1) [17]. In the UK, 61% of the adult population are either overweight (36%) or obese (26%) making it the European country with the highest obesity rate (Figure 1.1.2) [18]. Moreover, the prevalence of morbid obesity (BMI \geq 40 kg/m²) has been the fastest growing category of obesity in which it has tripled since 1993 [19]. The centres for disease control and prevention (CDC) in the United States have also shown a striking increase in overweight and obesity rates. Among US adults, the most recent data confirmed that 32% and 39.8% are overweight and obese, respectively [20]. The World Health Organization (WHO) has also pointed out the alarming overweight and obesity rates in the Middle East countries. Saudi Arabia for example, has the highest rates of overweight and obesity in the Middle East with a prevalence of 35.4% obesity and a further 34.6% overweight [21].

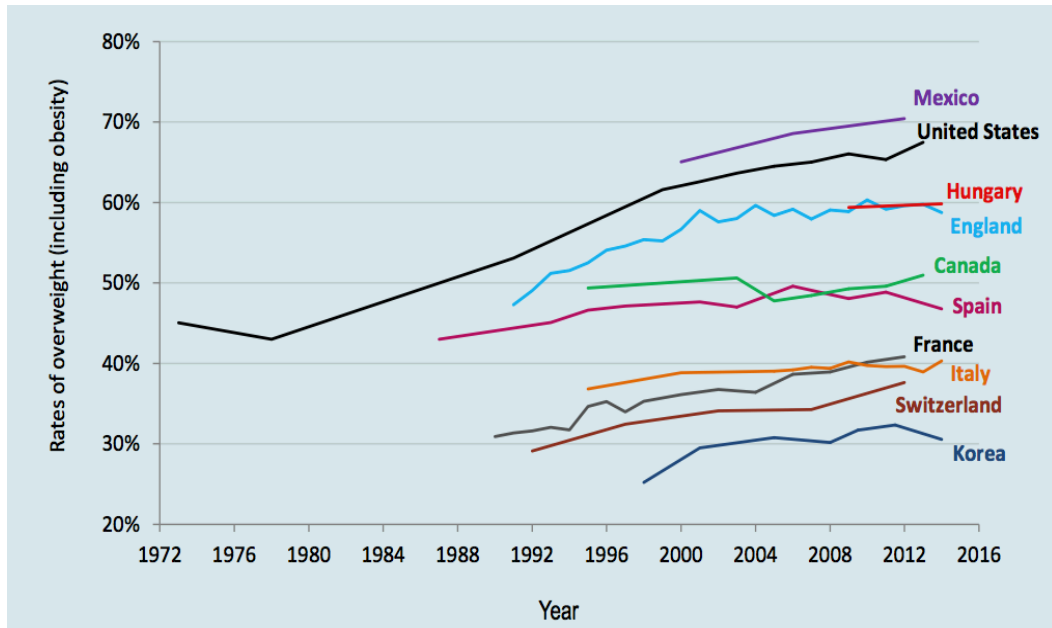


Figure 1.1.1 Increased overweight and obesity rates in adults aged 15-74 years: From the Organisation for Economic Co-operation and Development(OECD) 2017 Obesity Update [17]

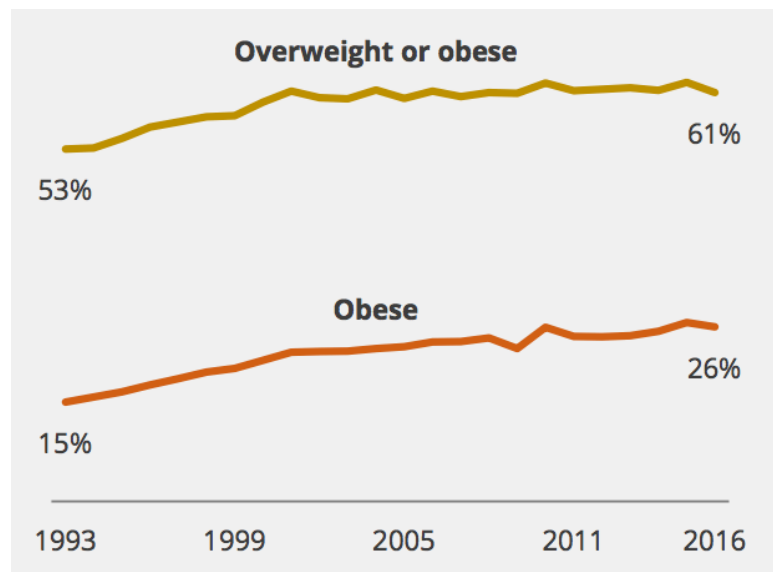


Figure 1.1.2 UK Obesity levels have increased from 15% to 26% since 1993: From House of Commons Library Obesity Statistics [18]

1.1.3 Obesity future projections

Recent projections from the organisation for economic co-operation and development (OECD) report a steady rise in obesity levels until at least 2030 (Figure 1.1.3). The United States, Mexico and England are expected to show a steady increase in Obesity rates, where 47%, 39% and 35% of the population, respectively are expected to be obese in 2030 [17]. The rise in overweight and obesity levels in the UK is predicted to increase costs to the NHS from 6.3 billion pounds per year in 2015, to 8.3 and 9.7 billion pounds per year in 2025 and 2050, respectively [17]. If the cost of obesity-related comorbidities is included, the cost is expected to rise from 19.5 billion pounds per year (in 2015) to 21.5 billion pounds per year by 2025 and 22.9 billion in 2050 [22].

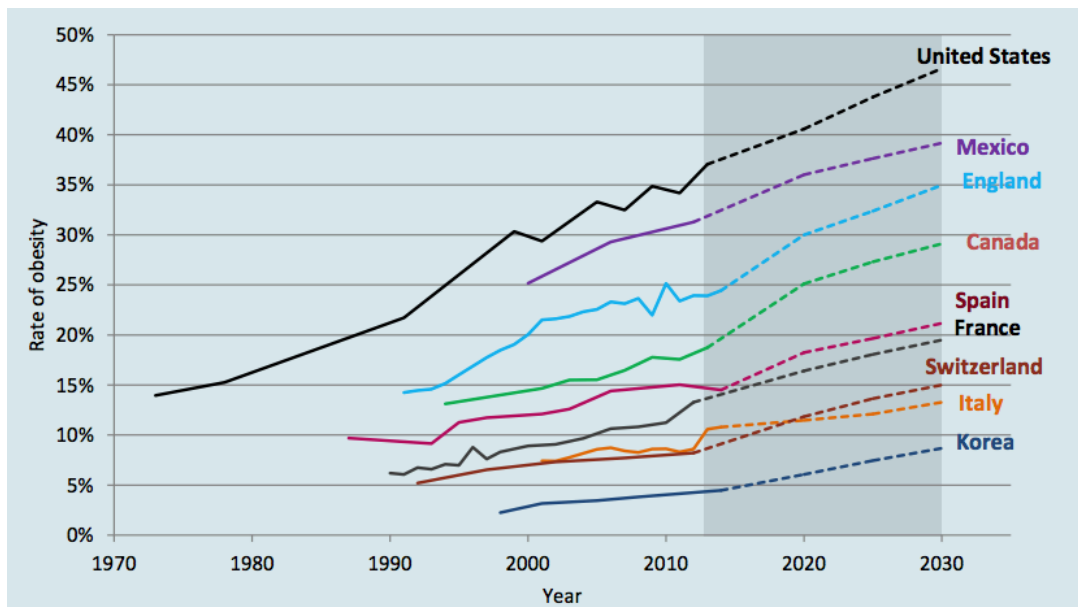


Figure 1.1.3 Projected future rates of obesity: From the Organisation for Economic Co-operation and Development s(OECD) 2017 Obesity Update [17]

1.1.4 Etiology of obesity

Several factors are associated with the etiology of obesity including: metabolic factors, dietary factors, genetic and appetite control factors, and physical inactivity. These factors have been studied extensively and are believed to result in energy imbalance and excessive fat deposition [23]. Although genes play an essential role in regulating body weight, the WHO Consultation on obesity concluded that dietary and environmental factors (*i.e.*, excess energy intake combined with sedentary lifestyles) are mainly responsible for the dramatic increase in obesity rates during the past 20 years [24]. Therefore, paying attention towards sufficient intervention efforts on dietary intake and physical inactivity can play an important role to tackle the prevalence of obesity in modern society.

Metabolic factors

Energy expenditure: resting energy expenditure, thermic effect of food, and activity-related energy expenditure are the three main components of total energy expenditure. Genetic factors appear to affect resting energy expenditure [25-27], thermic effect of food [26], and body fat adaptation to long-term overfeeding [28, 29]. Each component contributes differently to the daily energy expenditure, with the thermic effect of food being the smallest, accounting for about 10% of the total. Although several studies have documented that there is a reduced thermic effect of food response in obese individuals, the potential energy “savings” is probably less than 25 Kcal/day [30, 31], an amount that could explain a total weight gain of up to 2 kg [32]. The resting energy expenditure is considered the highest contributory factor to daily energy expenditure representing about

60% contribution. This factor depends mainly on body mass, particularly lean body mass, which is more metabolically active than fat tissue [33, 34]. On an absolute basis, obese individuals have higher resting energy expenditure because of their greater body size, including lean body (fat-free) mass. However, the values of resting energy expenditure in obese and non-obese persons tend to be comparable when adjusted for differences in fat-free mass [25, 27, 30-33]. Even after accounting for body composition, there is discrepancy in resting energy expenditure among people [35]. Therefore, relatively low resting energy expenditure values may appear linked to weight gain. This possibility has been addressed by several longitudinal studies which concludes that such differences in resting energy expenditure have only a minor impact on one's tendency to gain weight [9, 35-38].

Unlike resting energy expenditure, a strong relationship has been shown between a relatively low total daily energy expenditure with the amount of weight gain [31, 35, 39]. These data suggest that low non-resting (*i.e.*, activity-related) energy expenditure might be the component of daily energy expenditure that predisposes to obesity. Furthermore, spontaneous activity related energy expenditure is considered the most variable component representing, about 30% of total energy expenditure [40-42]. For instance, the total daily energy cost of physical activity varies with 9-fold noted in a metabolic chamber, and almost 30-fold in normal-living conditions [43, 44]. Thus, although not confirmed in all studies [36], the available evidence suggests that reduced activity-related energy

expenditure could potentially contribute to the predisposition to obesity [31, 45, 46].

Dietary factors

Several studies have shown a relationship between dietary fat intake as a percent of total caloric intake and adiposity, although the association seems to be highly variable [47-57]. In contrast to such cross-sectional analyses, longitudinal studies have reported that dietary fat is a potent predictor for body weight patterns [47, 56]. According to the USDA Nationwide Food Consumption and the National Health and Nutrition Examination Surveys (NHANES), the average fat intake in the United States decreased by an average of 4% between 1977 and 1988 in males and females [58, 59]. Paradoxically, the reductions in average fat and energy consumption were associated with a progressive increase in obesity rates in the US adult population [60]. Similarly, while average recorded energy intake have substantially declined in the UK population between 1970 and 1990, obesity has become more common [61]. Admittedly, measuring dietary patterns outside carefully-controlled laboratory conditions is problematic perhaps because of the underreporting of actual food intake, particularly among obese individuals [62, 63]. However, several recent surveys have shown that food has shifted in ways that promote overeating: High calorie and high fat foods are not only affordable but also easily available (*i.e.*, vending machines of energy dense food and beverages in schools and offices, numerous fast food restaurants, *etc.*). These highly palatable foods are frequently available in large portion sizes, leading to increased daily caloric intake [64-68]. In addition to increased commercial portion

sizes, the number of processed food items (typically high in sugar, fat, and sodium) available in supermarkets, grocery stores, and convenience stores has rose steeply. Nowadays, the majority of products in supermarkets stores are pre-packaged foods, highly processed and non-perishable. These products are heavily advertised not only to adults but also to adolescents and children. Convenient, easy to prepare, and inexpensive, these high calorie products were found to prevent people from consuming fruits and vegetables and nutritious foods [69]. Therefore, the current evidence recommends a healthy diet should limit or even exclude all but traditionally processed foods and snacks; consisting mainly of fruit and vegetables, whole grains, legumes, and limited amounts of dairy, meat and eggs. However, according to the 2013 health survey for England, 41% of women and 45% of men are found to consumes less than 3 servings of fruit and vegetables per day [45].

Finally, it is evident that the increased use of the reduced-calorie food products and the concurrent reduction in energy and fat intake have not attenuated the increased prevalence of obesity. Thus, if these dietary intake data are correct, then the average level of daily physical activity-related energy expenditure must be declining significantly [60].

Genetics and appetite control

Numerous studies have highlighted the involvement of several genes to predispose obesity in which a mutation in these genes may lead to sever obesity. These genes include: peroxisome proliferator-activated receptor- γ ,

melanocortin4 receptor, pro-opiomelanocortin, protein convertase 1 and thyroid hormone receptor β [70]. Other mutations such as a in the FTO gene were found to contribute to weight gain but not to the degree of obesity [71]. Despite the somewhat widespread belief that metabolic rate or selective conversion of excess calories into fat stores may be affected by such genetic pre-dispositions, disturbed hypothalamic pathways controlling hunger, satiety and food intake might be affected by monogenic defects that are associated with human obesity [72]. Perhaps one of the better understood examples of genes involved in controlling energy intake and appetite is the leptin gene [73].

One of the traditional theories of the body's ability for appetite control involves signals that inform the brain about the state of AT stores. This theory has given rise to the notion of a lipostatic mechanism which states that circulating factors are produced in proportion to the body fat stores. These factors (products) act as signals to the brain and initiate changes in energy intake and expenditure [74, 75]. In 1994, Zhang Y *et al.* and his colleagues [76] described the leptin gene within AT which provided a molecular basis for this theory. The main role of its product in the circulation, leptin, is to suppress food intake and to increase energy expenditure in animals through its signals to the brain (Figure 1.1.4). However, the genetic link between leptin and obesity been reported in only morbidly obese individuals [77, 78]. A number of studies have identified leptin deficiency in severely obese children and adults due to a mutation in the leptin gene[79-82]. Moreover, treatment with recombinant leptin led to a significant decrease in body weights in adults and a nine-years old girl [80, 81]. Contrary to early

expectations that deficient leptin synthesis may cause obesity in humans (e.g., a result of failure to suppress appetite), fasting serum leptin levels are elevated in obese patients and, in most cases, alongside increased adipose tissue mass [83-85]. These findings led some researchers to suggest that obesity promotes leptin resistance by activating cellular processes that attenuate leptin signalling [86-88].

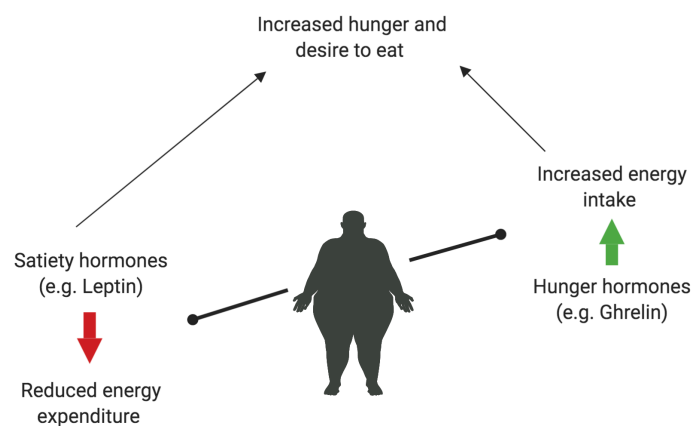


Figure 1.1.4 Imbalances between hunger and satiety hormones: Several factors including obesity may result in imbalances between energy intake and expenditure manifested by increased signals for energy intake (increase in the hunger hormone (e.g. ghrelin) and decrease in satiety hormones (e.g. leptin) leading to increased hunger and desire to eat and eventually to obesity (original figure drawn)

Physical inactivity

Data on trends in energy expenditure, have shown that the increased prevalence of obesity was parallel to reduction in physical activity levels and a rise in sedentary behaviours in various populations [89, 90]. A strong inverse correlation between physical activity level (PAL) and adiposity, with correlation coefficients in the order of -0.35 [91]. In 12,669 adult Finns, the prevalence of overweight was noted to be significantly higher in sedentary men (14%) and

women (21%) than in physically active men (7%) and women (8%) [92]. Generally, weight training exercise which is similar to aerobic exercise results in approximately 0.1 kg/week decrease in body fat [93]. Moreover, in a randomised control trial, women who lost weight through 6 months of diet and exercise, those who performed approximately 40 min/day (280 min/week) maintained their weight loss over an additional 12 months [94]. However, the amount of energy expended varies according to the type of physical activity performed. Furthermore, the energy physical activity (energy expenditure/work performed) is modified by the muscle groups used, relative intensity, and the range of movement involved [95-97]. For instance, to perform the same amount of bicycle work at a high intensity, 22% more energy is required than that of a low intensity [98], and three times as much energy is needed to make one bench press at 80% of one repetition maximum compared with four bench presses at 20% of maximum [99]. Therefore, some activities might be more beneficial than others in respect of their impact on energy balance.

1.1.5 Obesity and adipose tissue

Obesity is characterized by increased adipose tissue mass which is determined by adipocyte volume and cell number [100]. The volume of adipocytes is regulated by storage and removal of triglycerides [101]. Triglyceride removal rate is known as lipid turnover which refers to lipid removal from adipose stores through a process called lipolysis (hydrolysis of triglycerides) followed by the irreversible oxidation process. Lipid age can be estimated by measuring nuclear bomb test-derived ¹⁴C in adipocyte lipids and a high lipid age is an

indicator for low lipid turnover. Lipid age is not associated with adipocyte size, individual's age, or gender [102]. However, lipid age and hence lipid turnover rate is affected by obesity as triglyceride removal rate is reduced during this condition. Furthermore, lipid turnover is inversely associated with insulin resistance [103].

Obesity is related to reduced lipid turnover rate and increased triglyceride storage [102]. In obesity, blunted lipolysis appears to be independent of fat mass as it has been shown to be present in non-obese first-degree relatives of obese individual [104] and in obese insulin-resistant subjects after their weight loss [105].

In addition to adipocyte volume, the number of adipocytes is also a determinant of adipose tissue mass. In adulthood, adipocytes undergo a constant state of flux as preadipocytes are converted to lipid-filled mature adipocytes and older adipocytes endure cell death. Approximately, 10% of adipocytes are estimated to be renewed annually in adults independent of BMI. However, despite this constant turnover, adipocyte number is kept constant in normal BMI [106] but rises with obesity [100]. Obese individuals have greater number of adipocytes compared with non-obese subjects. Even after major weight loss post bariatric surgery, the number of adipocytes in the obese individuals remains the same although the size decreases [106].

1.1.6 Adipose tissue and inflammation

The growing global concern about the rapid rise in obesity rates has led to an emphasis on the “undesirability” of white adipose tissue (WAT). However, WAT

plays several vital roles in mammalian physiology. The traditional view of the WAT is that it functions to provide long-term fuel reserves and during food deprivation releases fatty acids for oxidation in other organs. Therefore, during periods of positive energy balance the size of adipose tissue stores increase whereas it decreases during periods of higher energy expenditure. Moreover, WAT can provide thermal isolation, and this is especially evident in the case of the blubber (thick layer of vascularized adipose tissue) of marine mammals such as seals and whales [103, 107].

In addition to long-term fuel reserve and thermal insulation, additional functions have recently attributed to WAT including the role in inflammatory processes through preadipocytes acting as macrophage-like cells [108] and a role in glucose homeostasis (Figure 1.1.5). Glucose homeostasis was confirmed by genetically modified mice containing little WAT; these lipodystrophic animals became diabetic, and exhibited both hyperglycaemia and substantial hyperinsulinemia [109, 110].

Adipose tissue is primarily composed by preadipocytes and mature adipocytes, but other types of cells constitute the stromal-vascular fraction including macrophages, lymphocytes, endothelial cells, fibroblasts, and neutrophils [111]. The presence of these cells account for the vast release of molecules known as adipokines with several important physiological functions, many of them involved in metabolism and others are involved in the balance between pro- and anti-inflammation to maintain the homeostasis. In obesity, adipose tissue exhibits a strong inflammatory response, which is one of the most

important factors that results in the development of insulin resistance and the pathogenesis of T2DM [112].

Adipocytes are metabolically active cells that produce and secrete several hormones which regulate whole body homeostasis including: leptin, resistin, adiponectin, and visfatin. These hormones are identified to play an important role in the regulatory functions of several mechanisms including: fatty acid synthesis, energy metabolism and beta-oxidation [113, 114]. As previously mentioned, food intake is suppressed by the hormone leptin. The metabolism of glucose and fatty acids is regulated by adiponectin. Resistin is a hormone implicated in decreasing the response to insulin, and visfatin is a hormone which is involved in the utilization of glucose. However, these hormones are also involved in inflammatory processes and can interact with cells of the innate and adaptive immune system regulating the production and release of cytokines from adipocytes and the stromal-vascular fraction[115, 116].

Many cytokines are implicated in inflammation with differences in type of cytokine according to the person metabolic health status. They can be classified as anti-inflammatory and pro-inflammatory cytokines depending on the condition with some displaying both anti- and pro-inflammatory functions. Moreover, depending on the condition of the body types (lean, overweight or obese), cytokines can also be produced; for instance, IL-4, IL-10, IL-13, and IL-1 receptor antagonist (IL-1Ra) are commonly produced in the adipose tissue of lean subjects, and exert anti-inflammatory roles. In contrast, IL-1, TNF α , and IL-6 which are implicated in inflammatory process are mainly produced and secreted in obese

conditions [117, 118]. Other cytokines that can function as anti-inflammatory and pro-inflammatory mediators are known to be produced by the adipocytes including: , interferon (INF)- α , Leukemia inhibitory factor and transforming growth factor (TGF)- β [119].

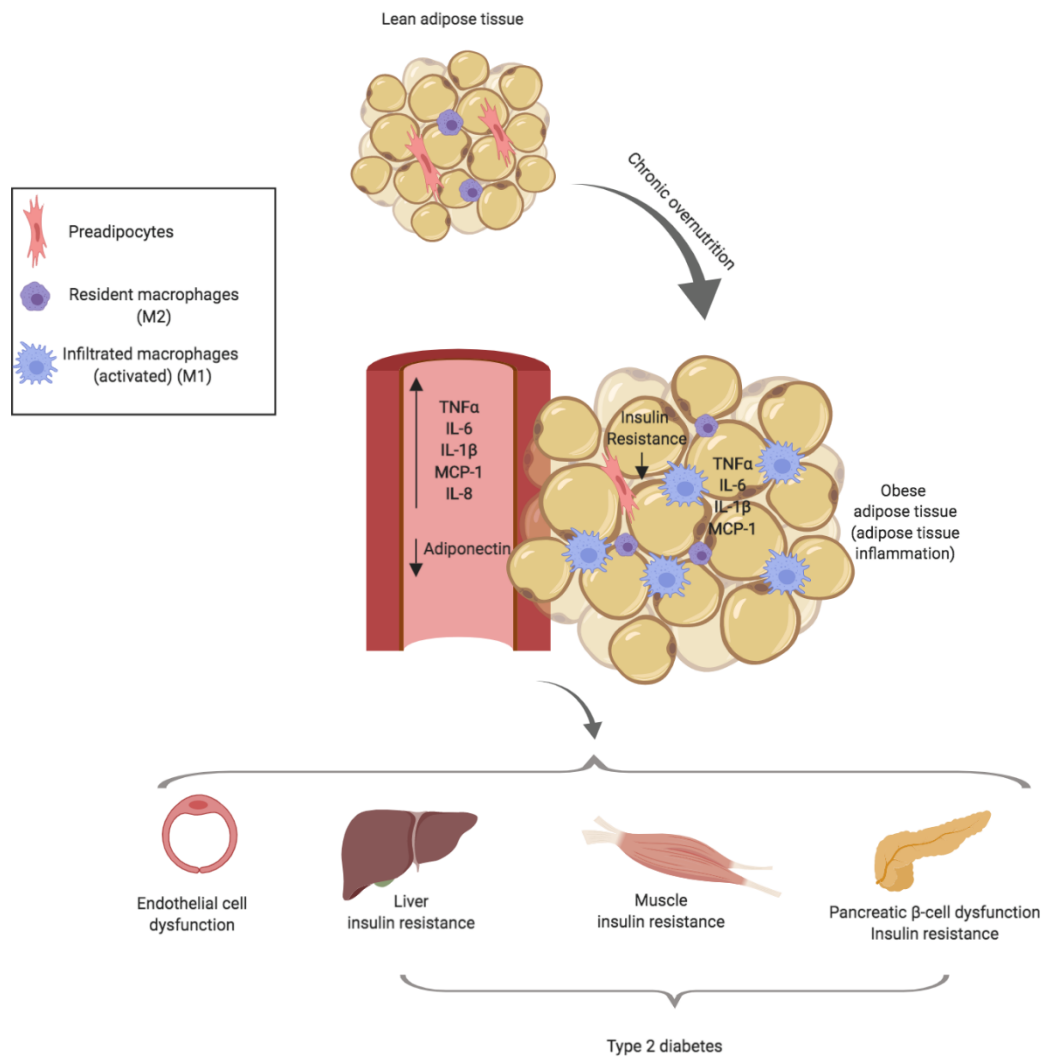


Figure 1.1.5 Obesity-induced macrophage infiltration into adipose tissue causes insulin resistance: Chronic overnutrition leads to disruption of local signalling networks, in addition to impaired adipogenesis and adipose expandability. This, in turn, results in adipocyte hypertrophy and dysfunction. In lean adipose tissue, M2 macrophages are distributed evenly throughout the tissue. Adipokines such as adiponectin and other anti-inflammatory mediators are increased. In obese adipose tissue, M1 macrophages increase their number by both infiltration and local proliferation. M1 macrophages secrete proinflammatory cytokines. Proinflammatory adipokines production such as TNF α , IL-6, IL-1 β , and MCP-1 increases. In addition to inflammatory cytokine secretion within adipose tissue and into the circulation, ectopic lipid accumulates in extra-adipose sites (e.g. liver and muscle), which together, contribute to the pathogenesis of insulin resistance by acting as insulin resistance-inducing adipokines and eventually leading to T2DM. (original figure drawn)

1.1.7 Relationship between obesity and inflammation

Inflammation is a process of modulation of innate immune system, in response to exogenous and endogenous stimulants, such as tissue stress, injury and microorganism infection [120]. The response to inflammation consists of four main components: (1) exogenous or endogenous factors, such as molecular patterns associated with damage and pathogens, which are derived from bacteria, viruses, parasites, fungi, and cell damage, in addition to toxic cellular components or any other harmful conditions [121]; (2) Cell-surface receptors which recognize these molecular patterns, including Toll-like receptors (TLRs), retinoic acid inducible gene (RIG)-like receptors and nucleotide binding oligomerization domain (NOD)-like receptors (NLR) [122, 123]; (3) pro-inflammatory mediators, such as chemokines and cytokines [124]; (4) and the pro-inflammatory mediators target cells and tissues [125].

Adipocytes and macrophages of the stromal fraction of adipose tissue express elevated levels of pro-inflammatory cytokines, such as nuclear factor (NF)- κ B resulting in an increase of TNF α , IL-6, and IL-1 β [126]. This is associated with the development of obesity and obesity-related insulin resistance, since inflammation causes a considerable deterioration in the insulin and leptin signalling pathways [126-129].

The changes in metabolic syndrome as a result of obesity are one of the most common factors that evoke the activation of inflammation, producing other alterations such as oxidative stress and cellular hypertrophy, among others [130].

The immune and metabolic systems are made up of hormones, cytokines, transcription factors, bioactive lipids and signalling proteins. Therefore, the basic inflammatory response promotes a state of catabolism and inhibits anabolic pathways such as the potent and highly conserved insulin signalling pathways [130, 131]. The inflammation caused by obesity is mostly the result of excessive fat consumption and macro nutrients causing deposition in other organs, mostly in the liver, changing insulin levels. Changes in respiratory rate, blood pressure, heart rate, and psychological factors are other conditions that are attributed to obesity [131, 132] (Figure 1.1.5).

1.1.8 Obesity and the mechanism of insulin resistance and T2DM

Although many of the detailed mechanisms linking the expanded fat tissue that defines obesity with the manifestation of insulin resistance remain unknown, the past several years have witnessed a vast increase in the understanding of the relation between adipose tissue hormones and insulin hormone which is currently referred to as the adipo-insular axis [133]. In addition to being caused by obesity, accumulating evidence considering the probability that hyperinsulinemia and insulin resistance, can contribute to the development of obesity [134]. Adipocytes are one of the most highly insulin-responsive cell types, and insulin is a critical promoter of many of vital aspects of adipocytes biology. Insulin regulates triglyceride stores in adipocytes by a number of mechanisms, including promoting the differentiation of preadipocytes to mature adipocytes, stimulating triglyceride synthesis (lipogenesis), stimulating glucose transporter GLUT4 transcription and translocation from the intracellular part to the cell surface, as well as inhibiting

lipolysis. Insulin also increases fatty acid uptake, derived from circulating lipoproteins, by stimulating the activity of lipoprotein lipase in adipose tissue [134]. The metabolic effects of insulin are mediated by various signalling cascades, initiated by insulin binding to its receptor IR of the target tissue which in turn elicits the phosphorylation of the receptor, stimulating the activation of tyrosine kinase receptor and consequently eliciting the tyrosine phosphorylation of insulin receptor substrates (IRSs) [134, 135]. The phosphorylation of IRS promotes the activation of phosphatidylinositol 3-kinase (PI3K), subsequently, AKT/protein kinases B and C are activated/phosphorylated [136, 137]. Phosphorylated/activated AKT initiates GLUT4 translocation from the intracellular site to the cell surface to transport glucose into the cell [138] (

Figure 1.1.6).

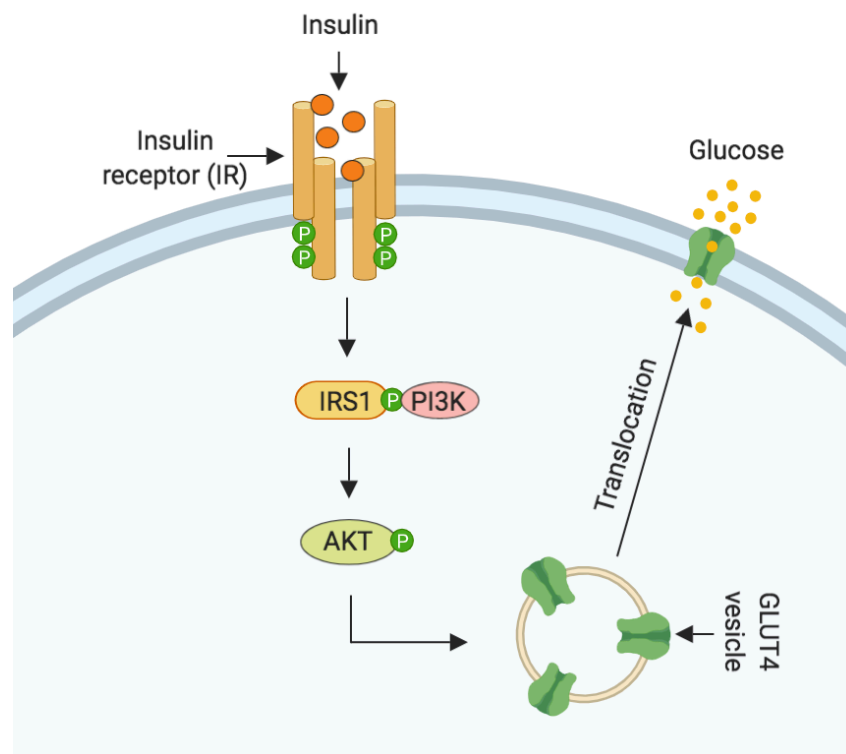


Figure 1.1.6 Insulin signalling pathway: In this figure, 6 principal proteins involved in insulin signalling pathway been highlighted such as: insulin, insulin receptor (IR), insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), protein kinase B known as (AKT) and glucose transporter-4 (GLUT4). The metabolic effects of insulin are mediated by various signalling cascades, initiated by insulin binding to its receptor IR of the target tissue which in turn elicit the phosphorylation of the receptor, stimulating the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). The phosphorylation of IRS-1 promotes the activation of PI3K, subsequently, AKT/protein kinase is activated/phosphorylated. Phosphorylated/activated AKT initiates GLUT4 translocation from the intracellular site to the cell surface to transport glucose into the cell. (original figure drawn)

In obesity, where adipose tissue is expanded, these pathways are dysregulated (Figure 1.1.7), leading to decreased response to insulin in skeletal muscle and fat tissue, causing elevations in lipid and blood glucose concentrations and increased insulin secretion by pancreas leading eventually to what is called insulin resistance [139]. In clinical definition, insulin resistance refers to abnormal higher levels of insulin to maintain blood glucose at normal levels, while at the cellular level, insulin resistance refers to the defects of insulin signalling pathways

[140]. Chronic elevated insulin levels might result in poor insulin response or sensitivity and finally causes disruption of the balance between β -cell functions and the peripheral tissue response to insulin which ultimately leads to the manifestation of T2DM [141]. Adipose tissue is now believed to have significant roles in modulating metabolism through its functions in glucose uptake making obesity a potent causative factor for insulin resistance [142].

Over the past several years, insulin resistance been defined as a state of low-grade inflammation which indicates that inflammation is a major contributing factor for its development [127, 143-146]. Within the adipose tissue microenvironment, adipocytes produce adipokines, such as leptin, adiponectin, and resistin to regulate systemic lipid and glucose metabolism (5, 6). However, during obesity, immune cells such as macrophages and T cells are progressively infiltrated into adipose tissue being the major source of inflammatory cytokines, including $\text{TNF}\alpha$, IL-6, IL-1B, MCP-1 and IL-8 [127]. These cytokines act through classical receptor-mediated processes to stimulate both the mitogen activated protein kinases (MAPKs) and the I κ B kinase- β (IKK β)/nuclear factor- κ B (NF- κ B) pathways, resulting in upregulation of potential inflammatory mediators that can directly and indirectly lead to insulin resistance [147-149] (Figure 1.1.7).

In mammals, there are three major classes of MAPKs, including the extracellular-signal-regulated kinase (ERK) 1/2, C-Jun N-terminal kinases (JNKs) and p38 MAPK are another important intracellular signalling pathways that play a vital role in modulating, insulin sensitivity and the pathogenesis of diabetes. A study showed that ERK1/2 knockout mice were resistant to diet-induced insulin

resistance due to impaired adipogenesis [150]. Another study showed abnormal elevated levels of activated JNK during obesity in insulin target tissues, including the liver, skeletal muscle and adipose tissue [151]. JNK deficient mice exhibited improved insulin signalling and were protected against obesity-induced insulin resistance. Ser-307 of insulin-receptor substrate 1 (IRS-1) is an inhibitory phosphorylation site targeted by JNK. TNF α inhibits the signalling capacity of the insulin receptor and induces Ser-307 phosphorylation through JNK [151, 152]. Moreover, several studies have demonstrated that p38 MAPK plays a crucial role in the modulation of hepatic glucose production. Besides stimulating hepatic glucose production, p38 MAPK activation reduces lipogenesis, and triggers apoptosis. Furthermore, all aspects of the inflammatory pathways mostly involve p38 MAPK activation/phosphorylation. Recent evidence indicates a key role of p38 MAPK in the pathogenesis of endothelial dysfunction and insulin resistance [153]. Thus, it is essential to tightly control MAPK pathways to prevent the development of metabolic disorders (Figure 1.1.7).

1.1.9 Regional distribution of adipose tissue

Regional differences in the distribution of adipose tissue in addition to its lipid-handling capacity is a better predictor for metabolic risk than overall adiposity. Large-scale prospective epidemiological studies have reported a stronger association between metabolic disorders and waist circumference than BMI [154, 155]. Among obese subjects, inflammatory macrophage infiltration was higher in subcutaneous abdominal adipose tissue than visceral fat depot and was strongly associated with metabolic disorders [156]. In addition, epidemiologic

studies revealed a strong relationship between subcutaneous fat tissue with metabolic risk [157, 158]. Moreover, in individuals suffering from lipodystrophic syndromes, the loss of gluteo-femoral or subcutaneous fat results in increased lipid deposition in visceral WAT which eventually results in worsening metabolic risk parameters [159, 160]. Although the reason behind this risk discrepancy remains controversial, evidence suggests this might be at least explained through tendency for inflammation. Whilst subcutaneous WAT has a higher capacity for lipid handling [161, 162], visceral WAT has a higher supply of innate immune cells like macrophages that allow it to play a critical mechanical and protective role against external inflammatory stimuli [163]. The local innate immune cells in visceral WAT of individuals with normal BMI and who are metabolically “healthy” are mainly M2 “repair” macrophages that produce and secrete anti-inflammatory molecules (such as IL-4 and IL-10) and play a primarily antigen presenting role [164]. However, with increased adipocyte apoptosis, the macrophages are distributed in the tissue and form a crown-like structures (CLS) and adopt an M1 phenotype (classically activated macrophages) which is capable of secreting pro-inflammatory molecules like IL-6, TNF α and IL1- β [165, 166] (Figure 1.1.5). The combination of the three factors - adipocyte apoptosis, CLS and circulating inflammatory cytokines - during obesity are strongly correlated with metabolic risk [166, 167].

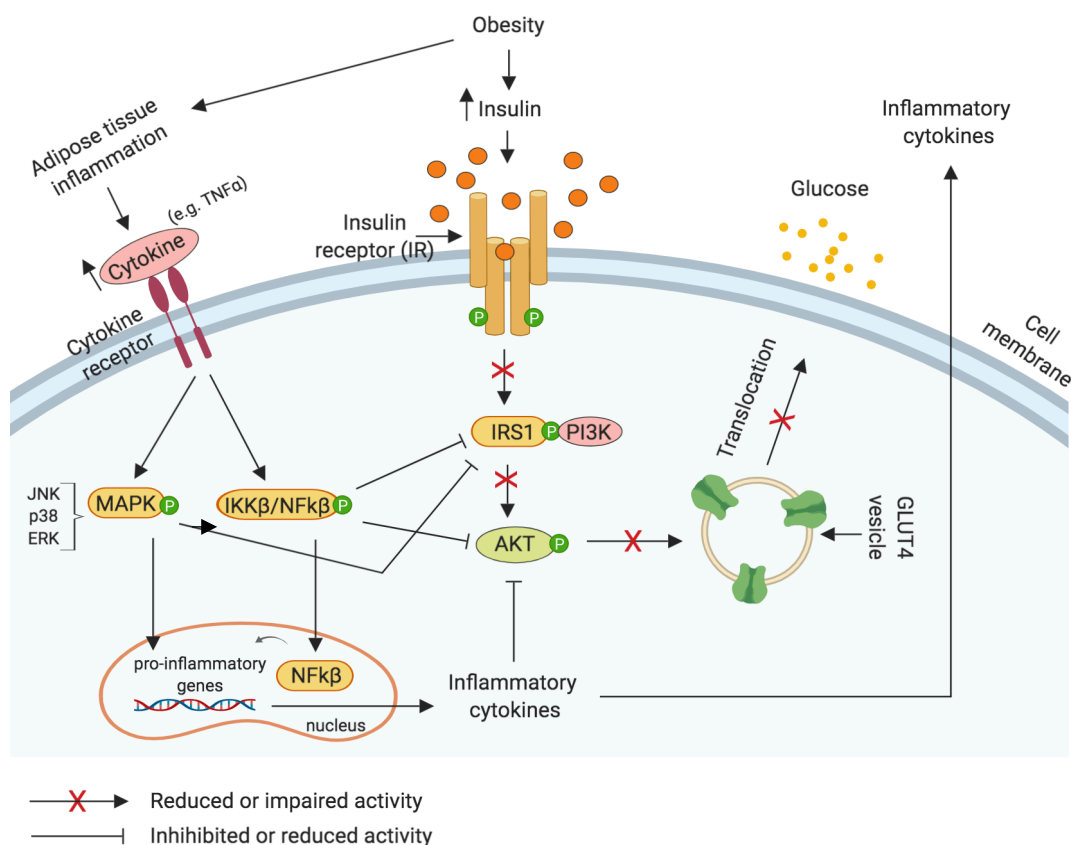


Figure 1.1.7 Insulin resistance pathway: In obesity, insulin levels increase and adipose tissue is expanded where inflammatory cytokines are secreted. When hyperinsulinemia is developed as a result of obesity, insulin binding to its receptor, receptor phosphorylation, and phosphorylation of IRS are reduced within target cells including adipocytes. The reduced IRS-1 expression, will result in impaired IRS-1–associated PI3K activity which in turn will reduce AKT phosphorylation and, hence, impair GLUT4 translocation. Activated immune cells (macrophages) within adipose tissue release inflammatory cytokines which induce insulin resistance via activation of NFκβ and MAPK pathways (JNK, p38 and ERK 1 and 2). Activation of these pathways may block insulin signalling and finally lead to the occurrence of insulin resistance. In addition, NFκβ and MAPK pathways are involved in the production of proinflammatory cytokines which may in turn become activation stimuli of these pathways. The consequent effects of hyperinsulinemia and inflammatory pathways may eventually lead to impaired or inhibited GLUT4 translocation which prevent glucose transportation into the cell. (original figure drawn)

1.1.10 The Malnutrition during Obesity: micronutrient deficiencies that promote insulin resistance or diabetes

One of the main causes behind the higher worldwide prevalence of obesity over the past four decades is the increased availability of high-calorie, nutrient-poor and low cost foods [168]. Modern agriculture and food production methods

led to a relative reduction in the micronutrient content of public foods [169]. Therefore, obese individuals tend to have relatively high rates of micronutrient deficiencies, despite their higher consumption of dietary sources [170, 171]. Certain micronutrients are known to act as cofactors in pancreatic β -cell function, glucose metabolic pathways and in the insulin signalling cascade which suggest that deficiency in these micronutrients may play a role in the development of insulin resistance and T2DM. Obese individuals have 4-fold higher risk to develop T2DM, although the causal association between obesity and diabetes is complex [172]. Increased insulin resistance, pancreatic β -cell dysfunction, incretin hormone resistance, oxidative stress, and behavioural and genetic factors all contribute to the development of diabetes in obese humans [173]. Deficiencies in specific micronutrient in obese subjects may also influence the development of T2DM [174].

The most common micronutrient deficiencies among obese individuals that are related to the development of diabetes are VitD, chromium, biotin, thiamine, B12, and the antioxidant vitamins including C and E [174]. However, VitD insufficiency is the most common among people with obesity and T2DM. The prevalence of VitD insufficiency (defined as <30 ng/ml) has been reported in 80-90% of obese individuals and 85-90% of patients with diabetes [170, 175]. A significant body of evidence suggests that VitD supplementation has beneficial effects on improving glucose metabolism and insulin signalling in patients with impaired glucose tolerance or T2DM [176]. Clinical studies have revealed that the role of VitD in insulin secretion and function includes the detection of VitD

receptor (VDR) in human pancreatic β -cells, the presence of 1- α - hydroxylase activity, and the responsiveness of insulin gene transcription to VitD in pancreatic β -cells [177-179]. VitD supplementation has restored pancreatic β -cell function when supplemented to VitD deficient animals [180-182]. Ample epidemiological studies reported an inverse association between circulating VitD levels and prevalence of T2DM [183-188]. Seasonal variations in glycaemic control in individuals with T2DM may also be due in part to variations in VitD levels, though behavioural differences may also explain these data [189, 190]. Recent meta-analyses and reviews of clinical trials suggest a beneficial effect of VitD supplementation on the development of T2DM in high-risk persons [176, 179, 191]. Furthermore, randomized control trials reported that VitD supplementation resulted in improvements in insulin sensitivity in subjects with insulin resistance or impaired fasting glucose [192-194]. A randomised trial has shown that 6 weeks of cholecalciferol (VitD₃) supplementation (120,000 i.u. every 2 weeks) was sufficient to improve oral glucose tolerance and insulin sensitivity in 71 obese individuals [192]. However, some studies have failed to show an effect of VitD supplementation [195, 196]. These studies have used only low doses of VitD (400 i.u. daily) which may not have been sufficient as the majority of participants (89%) did not achieve normal levels of VitD by the end of the trial [195]. Based on previous studies' data, VitD supplementation may be beneficial for patients at risk for development of T2DM. In addition, improvements in glycaemic control have been observed in patients already diagnosed with T2DM when VitD was administered [196-199]. In another trial, 90 patients with T2DM were divided

into 3 groups to consume plain yogurt drink, VitD–fortified yogurt drink (1000 IU VitD), or VitD + calcium–fortified yogurt drink (1000 IU VitD and 250 mg Ca/250 mL) daily for 12 weeks. Results showed that among patient who consumed either VitD only or VitD + calcium–fortified yogurt the mean fasting glucose decreased by 13 mg/dl, mean glycosylated haemoglobin (HbA1c) decreased by 0.4%, improved insulin sensitivity, and 1-2 kg weight loss. No changes in diabetes markers been observed among patients who were on plain yogurt drink [198]. The use of VitD supplementation particularly for improvements in insulin sensitivity remains under debate. Given the high prevalence of VitD insufficiency in obese individuals and the observed beneficial effect, clinicians may consider VitD supplementation in this group.

1.2 VitD signalling in adipose tissue

1.2.1 VitD: an overview

The term VitD refers to two substances that are highly lipophilic: cholecalciferol (VitD₃) and ergocalciferol (VitD₂). VitD₃ is actually a steroid hormone and is not a vitamin, because it is synthesized from 7-dehydrocholesterol present in the skin under the effect of 270–290nm wavelength solar ultraviolet B radiations. Cholecalciferol or VitD₃ is barely active (*i.e.* , cannot perform the known biological functions of VitD). In order to perform its biological functions, VitD₃ needs to be converted into the biologically active 1 α ,25-dihydroxyvitamin D₃ (1,25 (OH)₂D₃) or calcitriol (Figure 1.2.1). In 1968, the first hydroxylated VitD metabolites were discovered by Blunt J. *et al* [200]. A small number of cytochrome P450 (CYP) enzymes are known to be responsible for all

activation and degradation steps and are collectively called VitD hydroxylases (will be discussed in following sections). The main circulating form of VitD is the 25 hydroxyvitamin D₃ (25(OH)D₃) which is a precursor of the active steroid hormone 1,25(OH)₂D₃. Kidney, is the main organ that controls the systemic 1,25 (OH)₂D₃ levels, synthesis and degradation. The VitD receptor (VDR), a transcription factor belonging to the nuclear receptor subgroup, is regulated by its natural ligand 1,25(OH)₂D₃ [201]. VitD is able to induce its own catabolism by upregulation of 1,25 dihydroxyvitamin D₃ 24 hydroxylase (encoded by the gene CYP24A1), this enzyme degrades VitD and all its metabolites. Vitamin D₂ or ergocalciferol can be taken up with the diet or supplements and is derived from ergosterol and is synthesized in yeasts and plants [202]. Vitamin D₂ undergo similar metabolic pathways as with vitamin D₃, it is hydroxylated to 25(OH)D₂ and 1,25(OH)₂D₂, respectively (Figure 1.2.1) [203]. Although these two are metabolized similarly, the potency of each of the two VitD types is different. In a randomized controlled trial, ingested vitamin D₃ was approximately twice as potent in increasing and maintaining serum 25(OH)D levels as vitamin D₂. However, the superiority of vitamin D₃ is under debate because different dose regimens have been used in previous studies [204, 205].

1.2.2 VitD metabolism

CYP enzymes are also known as VitD hydroxylases and all steps of the VitD metabolism from synthesis to degradation are controlled by them [206]. VitD hydroxylases are mainly located in mitochondria or microsomes [207]. The major circulating form of VitD, 25(OH)D₃, is synthesized by hydroxylation of

cholecalciferol on position C-25 by 25-hydroxylases which are mainly found in the liver. This is considered as the first step for VitD conversion into its active form (circulating form). The main VitD 25-hydroxylase has not been extensively studied, this orphan microsomal CYP, encoded by the gene CYP2R1 is highly conserved and substrate-specific microsomal enzyme and the expression levels of CYP2R1 gene are associated with circulating 25(OH)D₃ concentrations. Other CYP enzymes include CYP3A4, CYP27A1 and CYP2J2 which are responsible for 25-hydroxylase activity in human [208-212]. In the kidney, the second and the activation step takes place and 25(OH)D₃ is converted to 1,25(OH)₂D₃ (calcitriol) which is the active form (Figure 1.2.1). The hydroxylation on position C-1 α is completed by the 25-hydroxyvitamin D₃ 1 α -hydroxylase (CYP27B1) [206]. Finally, 1,25(OH)₂D₃ has the ability to self-regulate its degradation by inducing the expression of 24-hydroxylase (CYP24A1) that converts 25(OH)D and 1,25(OH)₂D₃ into inactive metabolites (*i.e.*, 24,25(OH)₂D and 1,24,25(OH)₃ VitD), which are further catabolized into inactive calcitroic acid [213]. This final step is tightly regulated, therefore, physiologic VitD toxicity is unlikely to result from sun exposure or even from the ingestion of very large amounts of dietary sources [214, 215]. Serum 25(OH)D₃ has a half-life of between 2-3 weeks [216] and levels below 10ng/ml or 25 nmol/l are considered as VitD deficiency and serum levels in the range of 10-20 ng/ml or 25-50 nmol/l are defined as VitD insufficiency (Figure 1.2.1) [217-219].

In addition to the adipose tissue role in synthesizing classical steroid hormones (e.g. androgens and estrogens), cytochrome P450 (CYP) enzymes were

also observed to be expressed in adipose tissue and appear essential to support activation of a variety of metabolic pathways, including the synthesis and metabolism of several subclasses of steroids such as oxy-derivatives of steroids/sterols (e.g. oxysterol) and secosteroids (e.g. VitD) [206, 220, 221]. Several studies have identified three of these enzymes: CYP2R1, CYP2J2 and CYP27A1 to be expressed in adipose tissue [220] whereas the fourth enzyme CYP3A4 was not expressed in either SC or OM tissue as shown in different studies [220, 222-225]. The presence of these CYPs in adipose tissue suggests the important role of VitD within adipose tissue and that lower expression of these enzymes in obesity may indicate an impaired VitD activation during obesity [220].

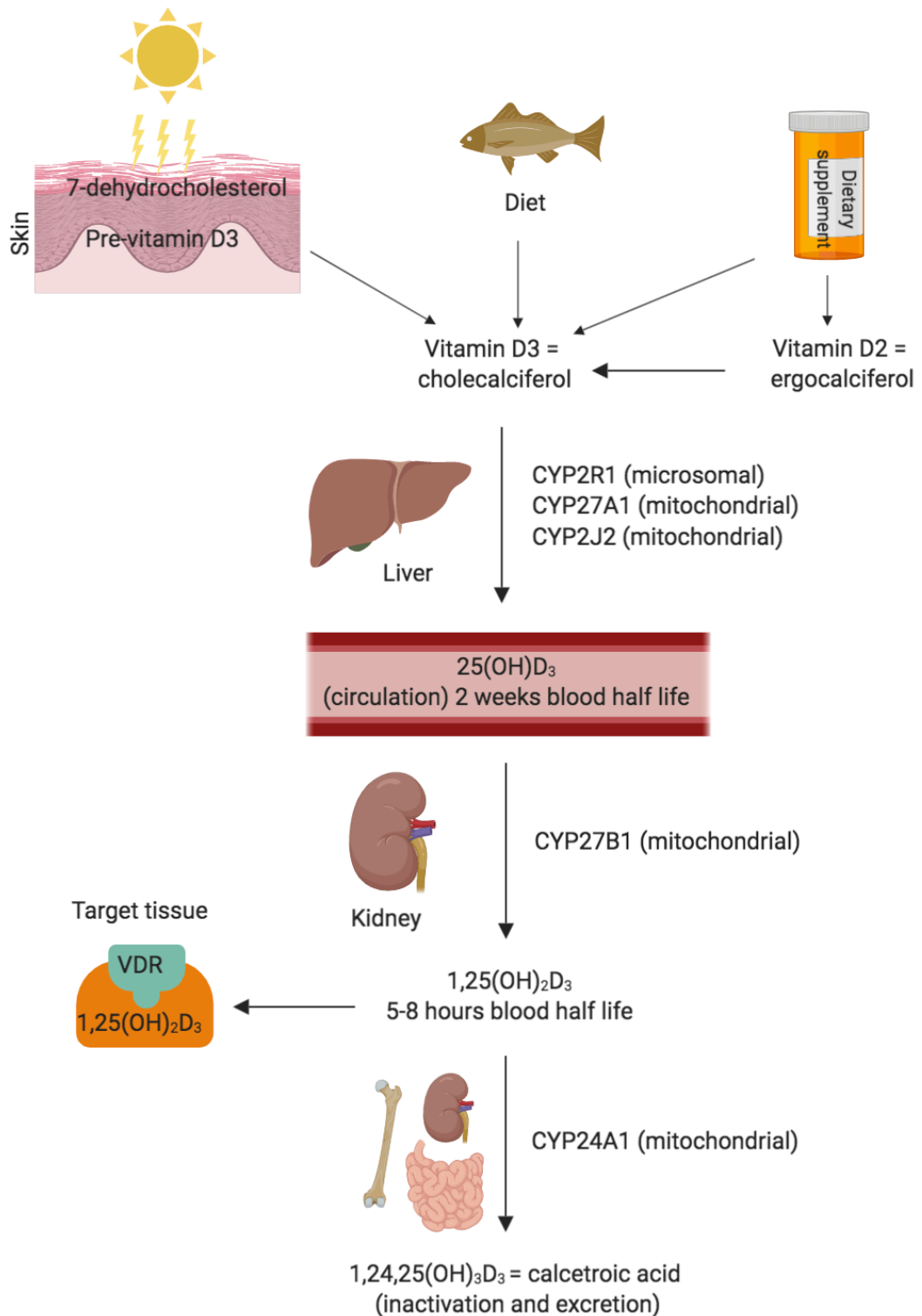


Figure 1.2.1 The metabolic pathway for VitD: Vitamin D3 (cholecalciferol) whether from sunlight exposure, diet or dietary supplements is converted in the liver to form the major circulating component of VitD the 25-hydroxyvitamin D₃ by the three 25,hydroxylases (CYP2R1, CYP27A1 and CYP2J2). In the kidney, the activation step takes place through the action of the 25(OH)D₃-1 α -hydroxylase, CYP27B1, to yield the active hormone, 1,25(OH)₂D₃ (calcitriol). The active hormone (1,25(OH)₂D₃) is transported to the kidney, intestine, bone, and other VitD target tissues where it binds to the nuclear VitD receptor (VDR) and performs its biological actions. Ligand-bound VDR upregulates expression of the 24-hydroxylase, CYP24A1, which inactivate and degrades 1,25(OH)₂D₃. (original figure drawn)

1.2.3 VitD receptor

The biological actions of $1,25(\text{OH})_2\text{D}_3$ are mediated through a member of the nuclear receptor superfamily, the VitD receptor (VDR), that regulates the transcription of many of VitD target genes (Figure 1.2.1) [226]. VDR, which has high affinity and specificity to VitD binds with $1,25(\text{OH})_2\text{D}_3$ then heterodimerises with a retinoid X receptor [227]. A genomic response is then generated when the heterodimer binds to VitD response elements in target genes [228, 229]. Moreover, there is also a plasma membrane VDR which mediates the rapid and acute actions of $1,25(\text{OH})_2\text{D}_3$ [230]. VDR has been reported in most human tissues, including in skin keratinocytes, osteoblasts, smooth muscle, macrophages, epithelial cells and pancreatic b-cells [231, 232]. The high expression of VDR in multiple tissue types may underlie the various effects of VitD and provide a mechanistic basis for the link between VitD deficiency and a number of diseases, including certain types of inflammatory bowel disease, cancer, CVD, diabetes (type 1 and type 2) and the metabolic syndrome[232-236].

1.2.4 Physiological Roles of VitD

For a long time, the endocrine function of VitD was believed to be confined to the metabolism of calcium and phosphorus [215]. However, VitD is now considered to be a multifunctional factor whose circulating concentration appears to be linked to the prevalence of several disorders including obesity [237] and metabolic disorders [191]. In addition to the kidney, several other tissues, such as the intestine, skin, immune cells and placenta, are able both to activate and degrade $1,25(\text{OH})_2\text{D}_3$ [238-240]. Autocrine and paracrine roles of the locally

produced $1,25(\text{OH})_2\text{D}_3$ include enhancing the innate immune system, decreasing autoimmune disease by modulating the adaptive immune system, lowering risk of heart attack and stroke, and exerting antitumorigenic activities [241]. Correlation studies of circulating $25(\text{OH})\text{D}_3$ levels and chronic disorders led to the conclusion that much higher levels of circulating $25(\text{OH})\text{D}_3$ are needed for these local autocrine/paracrine actions than for the endocrine actions of $1,25(\text{OH})_2\text{D}_3$ [242].

In case of certain illnesses, i.e., chronic liver failure, renal failure, hyperparathyroidism, or tumor-induced osteomalacia, that functionally impair VitD enzymes, the endocrine VitD metabolism is deregulated leading to VitD deficiency [215]. A meta-analysis from numerous epidemiological studies reported that low serum levels of $25(\text{OH})\text{D}_3$ cause osteomalacia and rickets and are associated with incidence and severity of different chronic diseases, such as osteoporosis, cardiovascular diseases, multiple sclerosis, metabolic syndrome, cancer of the breast, prostate, or colon [243, 244].

1.3 VitD, obesity and insulin resistance

1.3.1 The link between obesity and VitD

In clinical practice, VitD status is normally assessed by the measurement of plasma or serum level of $25(\text{OH})\text{D}_3$, the major form of VitD in the circulation [216, 245]. Numerous studies on VitD status have suggested an association between obesity and VitD deficiency as obese individuals tend to have low serum levels of $25(\text{OH})\text{D}_3$ [246-248]. This link was noticed when the development of

obesity epidemic was paralleled with VitD deficiency identification as a major global public health problem [249]. It is estimated that approximately 1 billion people worldwide have low circulating VitD levels (below 50 nmol/l) [215].

The direction of the relationship between obesity and low VitD still remains debatable due to the dynamic nature of the production and regulation of the active hormone [250]. However, several clinical and experimental studies have provided evidence for the role of obesity as a causative risk factor for the development of VitD deficiency [251]. Association studies have shown inverse correlations between serum 25(OH)D₃ levels with measures of obesity, including BMI, fat mass and waist circumference [252-255]. In particular, it has been estimated that each unit increase of BMI was associated with a 1.15 % decline of 25(OH)D₃ levels [256]. Furthermore, a strong inverse association been described between VitD status with both subcutaneous and visceral fat depots [254]. There is evidence that higher consumption of dietary VitD and increased serum 25(OH)D₃ levels are associated with lower visceral adiposity and omental adipocyte size in women undergoing gynaecological surgery [257]. Moreover, in a double-blind, placebo-controlled trial, dietary supplementation with a combination of VitD and Calcium for 4 months was related to beneficial reduction of visceral fat in overweight and obese individuals [258]. Consequently, low VitD might lead to adipose tissue accumulation and compromise normal metabolic functioning, and eventually contribute to the adverse health effects associated comorbidities, including insulin resistance and T2DM [259]. Fassina G. *et al.* [260] showed that large doses of vitamin D₂ resulted in increases in energy expenditure

due to uncoupling of oxidative phosphorylation in adipose tissues. However, data from clinical trials investigating the effects of VitD supplementation and increases in VitD status on body weight have not provided consistent results [50, 261]. In this context, it has been demonstrated that normalization of 25(OH)D₃ levels in subjects with VitD insufficiency with VitD supplementation could participate to prevent weight gain by reducing the 1,25(OH)₂D₃ production, likely through decreasing PTH levels [262]. *In vitro*, the effect of VitD and more specifically that of its active form, 1,25-dihydroxyvitamin D (1,25(OH)₂D₃), has been more extensively studied. It has been reported that 1,25(OH)₂D₃ inhibits adipogenesis, which may explain the relationship between obesity and VitD [263, 264].

Taken together, the current evidence strongly suggests that the VitD system is altered in obese individuals and this might have implications both for the development of obesity itself and of its related co-morbidities.

1.3.2 VitD system in adipose tissue

Despite limited data, it is widely recognized that adipose tissue is a pool for VitD in human subjects and rodents [255, 265-267]. Interestingly, the omental depot contains 20% more VitD than subcutaneous adipose tissue [220]. Heaney *et al.* calculated that 65% of total absorbed VitD in the body is in the form of cholecalciferol (vitamin D₃) which is inactive and 35% as the circulating form (25(OH)D). Approximately 75% of the cholecalciferol was in fat, while 25(OH)D was more evenly distributed throughout the body (35% in fat, 20% in muscle, 30% in serum, and 15% in all other tissues) [268]. As such AT may therefore contain

about 60% of total body VitD (cholecalciferol and 25(OH)D₃), but the amount of VitD stored in AT differs strongly between individuals and is not necessarily associated with serum 25(OH)D₃ levels [265], whereas 25(OH)D₃ fat tissue storage appears to be associated with 25(OH)D₃ plasma levels [269]. Other factors such as VitD status and amount of dietary consumption may also influence fat tissue content. Indeed, it has been calculated that when VitD intakes are low and when circulating 25(OH)D₃ level is less than 88 nmol/l, almost all VitD is converted to 25(OH)D₃ in the liver with very little amount deposited in tissues [105], indicating that low 25(OH)D₃ status is correlated with limited tissue stores as well [270]. In black American and Caucasian children aged 8-18 years old, VitD and 25(OH)D₃ were present in human fat tissue, and for the first time 1,25(OH)₂D₃ was reported to be also detectable in adipose tissue [255, 270]. In contrast to previous studies, this study has found that these molecules were located in adipocyte lipid droplets and that VitD and 25(OH)D content in adipose tissue were higher in lean than obese subjects, and suggested that the concentrations of these molecules in fat and serum might be affected by a large overall volume of distribution [255, 270].

A number of studies suggest that the metabolic components of the VitD system are evident in fat tissue, and VitD may be involved in the function of the tissue [224, 271, 272]. The gene expression of the CYP27B1, which encodes the CYP enzyme converting 25(OH)D₃ to 1,25(OH)₂D₃, has been shown in mouse 3T3-L1 preadipocytes and in adipose tissue of Wistar rats [224]. Moreover, Simpson – Golabii – Beymel Syndrome (SGBS) human adipocytes and preadipocytes [273,

274] and human mammary adipocytes [275] were noted to express CYP27B1 gene. The expression of CYP24A1 gene, which encodes the enzyme catalysing and degrading $1,25(\text{OH})_2\text{D}_3$, is also reported in murine 3T3-L1 adipocytes and by human adipocytes and preadipocytes [224, 274-276]. Therefore, adipocytes could be involved in the local synthesis and degradation of biologically active VitD.

Although gene expression of VDR has been reported in 3T3-L1 adipocytes and mouse white and brown adipose tissue [277, 278], very few studies have investigated whether VDR gene is expressed in human adipose tissue. Ding C. and his colleagues (2012), however, have observed that the VDR gene is expressed by human subcutaneous and visceral fat tissue [279] as well as by human cultured fat cells (preadipocytes and differentiated adipocytes) [274, 276]. Importantly, mature adipocytes are reported to secrete $1,25(\text{OH})_2\text{D}_3$ and were observed to contribute to higher levels of $1,25(\text{OH})_2\text{D}_3$ in conditioned media than human mammary epithelial cells; these cells are known to be highly efficient at activating $25(\text{OH})\text{D}_3$ [275]. These data suggest that mature adipocytes are capable to take up $25(\text{OH})\text{D}_3$, convert it to $1,25(\text{OH})_2\text{D}_3$ and then release the biologically active form to the local microenvironment – and possibly to the circulating pool (nevertheless, the kidney is the major source for the active VitD).

VitD metabolism is also known to take place in the macrophages, which have the ability to convert circulating $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$ [280]. The increase in fat tissue in obesity is accompanied with increased macrophage accumulation in the tissue [132, 166], which might enable the local hydroxylation of $25(\text{OH})\text{D}_3$.

However, the specific characteristics of macrophages in adipose tissue and their potential contribution to the local synthesis of $1,25(\text{OH})_2\text{D}_3$ is unknown.

Figures on the definite $1,25(\text{OH})_2\text{D}_3$ content of human fat tissue are very scarce. In a cross sectional study of 17 morbidly obese subjects undergoing bariatric surgery, concentrations of $1,25(\text{OH})_2\text{D}_3$ measured by liquid chromatography – MS (LC/MS) were much higher (more than 10-fold) in abdominal subcutaneous adipose tissue than in serum [281]. However, it is still not known whether the rise in $1,25(\text{OH})_2\text{D}_3$ concentrations in fat tissue is the result of increased uptake from the circulation or through local conversion in the obese state. Together, these data suggest that human fat tissue may well be a target for VitD, via endocrine and autocrine/paracrine actions of the hormone.

1.3.3 Possible mechanisms that may contribute to low VitD status during obesity

Perhaps, the most likely justification for low VitD levels in obesity is that, due to its lipophilic nature, VitD is largely sequestered in the fat tissue [37]. Nevertheless, a volumetric dilution associated with the greater volume of distribution of $25(\text{OH})\text{D}_3$ in tissue mass in obese subjects might be considered as a logical reason for the low circulating VitD levels [237]. However, many other possible mechanisms might account for the low VitD status. These include: lower dietary consumption of VitD by obese subjects, lesser exposure of skin to sunlight, as obese individuals tend to engage in less outdoor activity than non-obese subjects and impaired 25-hydroxylation and $1-\alpha$ hydroxylation in fat tissue [256]. In addition, weight loss surgeries could result in decreased intestinal absorption

due to bariatric or gastric bypass which induce a malabsorptive state [282]. Moreover, it has been suggested that fat tissue expansion in obesity may result from a state of excessive adaptive “winter response”, and that the decline in VitD skin production, due to less sunlight exposure, contributes to the tendency to increase adipose mass during winter [283]. Therefore, obese individuals have more storage capacity for VitD which leads to the lower levels [256]. Accordingly, obese individuals had a relatively smaller increase in serum VitD levels after either 24 h of UVB radiation or oral VitD supplementation compared to lean individuals [284]. The possibility that low VitD itself could contribute to obesity or reduce weight loss could be envisaged [285]. At low VitD levels secondary hyperparathyroidism is induced that results in elevated intracellular levels of ionic calcium in adipocytes [286]. This intracellular increase of calcium in adipocytes may increase fatty acid synthase expression, a potent modulatory enzyme in the deposition of lipids, and decrease lipolysis [287].

1.3.4 Role of VitD in adipose tissue inflammation

The potential role of VitD in regulating inflammation in obesity and other metabolic disorders has received increasing attention. Accumulated evidence has shown that $1,25(\text{OH})_2\text{D}_3$ has potent immunoregulatory effects, such as preventing the synthesis of inflammatory mediators by peripheral blood mononuclear cells from psoriatic patients [288]. It has been also reported that in human monocytes, the gene and protein expression of toll-like receptors (TLR) 2 and 4 (which allow the immune system to recognize inflammatory molecules) is downregulated by $1,25(\text{OH})_2\text{D}_3$ [289]. The production of lipopolysaccharide-induced IL-6 and $\text{TNF}\alpha$

was also reduced by either 1,25(OH)₂D₃ or 25(OH)D₃, perhaps by preventing MAPK activation in human monocytes/macrophages [290]. Furthermore, patients with T2DM reported decreased expression levels of TNF α , IL-8, IL-6, and IL-1 when 1,25(OH)₂D₃ was administrated [291]. *In vivo*, vitamin D₃ treatment has significantly reduced retinal inflammation and amyloid- β accumulation and improved the visual function in aged mice [292].

Adipose tissue inflammation, characterised by increased recruitment of macrophages and other immune cells in WAT, is a main pathological process in adipose tissue dysfunction [293, 294]. Several studies have demonstrated that macrophage-conditioned medium has significantly stimulated the secretion of proinflammatory cytokines (such as IL-8, IL-6 and MCP-1) from human preadipocytes and adipocytes; these molecules might induce inflammation, fibrosis and insulin resistance in adipose tissue [274, 295-298]. Moreover, in a dose dependent manner, 1,25(OH)₂D₃ has successfully reduced the protein release of TNF α -induced IL-6 and MCP-1 in human preadipocytes and adipocytes [299, 300].

The activation of NF κ B signalling pathway is essential for transcriptional induction of pro-inflammatory cytokines in several cell types including adipocytes [298, 301, 302]. Activation of NF κ B signalling is initiated by I κ B α protein degradation and translocation of p65 into the nucleus [303]. Gao D. *et al.* [299] however, demonstrated increased I κ B α protein abundance in human preadipocytes after preincubation with 1,25(OH)₂D₃. The preincubation with 1,25(OH)₂D₃, was noted to block NF κ B activation by stabilising I κ B α protein,

leading to an inhibition of p65 NFκβ nuclear translocation [304]. Generally, 1,25(OH)₂D₃ appears to be anti-inflammatory and it may downregulate macrophage-induced inflammation in fat tissue.

1.3.5 VitD hydroxylases in Inflammation

Little is known about the regulation of the VitD metabolising enzymes (VitD hydroxylases) during inflammation *in vivo*, although there are data suggesting that inflammatory mediators may increase local production of 1,25(OH)₂D₃ to block the inflammatory process [206].

Available *in vitro* studies have shown evidence that inflammatory cytokines stimulate the expression of VitD metabolizing enzymes. While the expression of renal CYP27B1 is tightly regulated by PTH and 1,25(OH)₂D₃, in extra-renal tissues, CYP27B1 is controlled independently of those mediators in a tissue-specific manner. Soluble factors such as cytokines and growth factors from the local environment affect cellular levels CYP27B1. IFN-γ, TNFα and IL-1 increase expression of this enzyme in immune cells [305]. TNF-α has been shown to induce the expression of both CYP27B1 and CYP24A1, with a greater extent for CYP24A1 [306]. CYP27B1 gene was highly expressed in patients with sarcoidosis, an inflammatory granulomatous disorder, and this increase has led to pathologic elevation of systemic 1,25(OH)₂D₃ levels and to hypercalcemia [307, 308].

Lipopolysaccharides (LPS), the ligand which activates the TLRs, can also increase CYP27B1 gene expression in dendritic cells (DCs) (antigen-presenting cells which are important for regulation of T cell-mediated immune response and

induction of immunological tolerance) and human macrophages [309]. The production of $1,25(\text{OH})_2\text{D}_3$ in monocyte-derived DCs is inhibited due to less active CYP27B1, while catabolism is not affected. Controlling VDR targets in these cells appears to occur in a paracrine manner. Kundu C *et al.* concluded that the $1,25(\text{OH})_2\text{D}_3$ secreted by macrophages is responsible for inducing expression of VitD target genes in the neighbouring DCs, reducing DC-dependent T-cell responses and inhibiting maturation of DC [310].

T-cell-derived cytokines modulate the expression of VitD hydroxylase CYP27B1 in monocytes via TLR2/1. In addition, IFN- γ boosted the activity of CYP27B1 and reduced that of CYP24A1 [306]. Mechanistic studies shown that IFN- γ -induced activation of CYP27B1 is regulated by NF κ B, and MAPK pathways [311]. Moreover, the activation of TLR1/2 by IFN- γ may increase the expression of CYP27B1 and VDR [309, 312].

1.3.6 The link between VitD and glucose homeostasis – possible mechanisms

Different mechanisms have been proposed by which $1,25(\text{OH})_2\text{D}_3$ may contribute to insulin secretion and sensitivity. The first suggested biological mechanism is the existence of VDRs and the expression of VitD metabolizing enzymes in the pancreatic β -cell along with the presence of a VitD response element in the human insulin gene promoter [177, 178, 313]. Aligned with this finding, a substantial reduction in insulin secretion in VDR knockout mice has been reported [314] and the human insulin gene has been found to be transcriptionally activated by $1,25(\text{OH})_2\text{D}_3$ [315]. VitD can indirectly affect insulin secretion by

modulating calcium flux via the cell membrane along with its role in the production and modulation of calbindin, a VitD-dependent Ca-binding protein in pancreatic β cells [316]. VitD might also protect β cells against fatal immune attacks, or programmed cell death either directly or indirectly by its influence on different components of innate and adaptive immune system at diverse levels. It has been demonstrated that $1,25(\text{OH})_2\text{D}_3$ might counteract apoptotic pathways and the inflammatory effect produced by increased cytokines via blocking of $\text{NF}\kappa\beta$ antiapoptotic protein [317].

Another possible mechanism elucidating VitD involvement in the pathogenesis of T2DM is the role of hypovitaminosis D in stimulating insulin resistance at the target tissues [318, 319]. The existence of the VDR in the cell membranes of target tissue, such as skeletal muscle, together with the upregulation of insulin receptors after treatment with $1,25(\text{OH})_2\text{D}_3$ seems to support this hypothesis [320]. Raised levels of parathyroid hormone (PTH) has also been proposed as a regulator for both insulin secretion and sensitivity by affecting glucose uptake and blocking insulin transport signalling in the target tissues mainly by elevating intracellular calcium concentration [321]. Insulin resistance can also be worsened by chronic inflammation and VitD might affect the inflammatory pathways via different mechanisms including regulation of the secretion of inflammatory mediators such as $\text{TNF}\alpha$, modulation of $\text{NF}\kappa\beta$ activity, downregulation of mRNA expression of TLR2 and TLR4 and regulation of genes encoding pro-inflammatory cytokines [322-324].

1.3.7 Link between VitD and glucose homeostasis - evidence from interventional and observational studies

Numerous clinical studies have related low VitD levels with the development of insulin resistance in adults [186, 325, 326] and children [327, 328]. Better β -cell function and lower glycaemia have been predicted by elevated basal levels of serum 25(OH)D₃ in subjects at risk for T2DM [329]. In mice, hypovitaminosis D impairs insulin secretion from the pancreas and decreases glucose uptake, which is partially restored following treatment with 1,25(OH)₂D₃ [330, 331]. Daily supplementation of cholecalciferol (2000IU) for four months has been shown to improve β -cell function in subjects at high risk of diabetes [197]. Moreover, data suggests that glycaemic control is significantly improved when serum 25(OH)D₃ levels have increased through daily intake of a VitD-fortified yogurt drink in patients with T2DM [198]. Thus, VitD may have an effect on insulin secretion from pancreatic β -cells, however further work in this area is required.

Ding, C. and colleagues [332] have demonstrated that 1,25(OH)₂D₃ reduced macrophage-induced inflammatory responses in human adipocytes by inhibition of ERK1/2 phosphorylation in a dose and time dependant manner. Moreover, the inhibition of p38 and ERK signalling during insulin resistance completely restored insulin-stimulated glucose uptake and insulin signalling in 3T3 adipocytes and muscle cells [333, 334]. These data suggest that the observed inhibitory effects of inflammatory processes exerted by VitD could be as a result of different molecular mechanisms where MAPKs are involved (Figure 1.3.1).

Furthermore, VitD has been shown to have beneficial effects on insulin sensitivity, as treatment with $1,25(\text{OH})_2\text{D}_3$ increased gene expression levels of insulin receptor and GLUT4 in U-937 promonocytic cells, perhaps through upregulation of phosphatidylinositol 3-kinase (PI3K) activity [315] (Figure 1.3.1). In addition to skeletal muscle and liver, adipose tissue is a key tissue exhibiting insulin resistance in obesity [335]. Although it only accounts for about 10% of total insulin-stimulated body glucose uptake, the decrease in insulin sensitivity of adipocytes increases the release of non-esterified fatty acids (NEFA) into the circulation, which might promote muscle and hepatic insulin resistance [336]. Treatment with $1,25(\text{OH})_2\text{D}_3$ has been shown to normalise insulin receptor levels and improve insulin response to glucose transport in epididymal adipocytes of streptozotocin-induced diabetic rats [337] (Figure 1.3.1). However, whether VitD metabolites have favourable effects on glucose transport and insulin secretion in human fat tissue remains to be clarified.

The beneficial effects of VitD have also been shown in prospective observational studies. Forouhi *et al.* [338] demonstrated that after adjustment for potential confounders, baseline $25(\text{OH})\text{D}_3$ levels were inversely correlated with fasting glucose, fasting insulin, and homeostasis model assessment of insulin resistance (HOMA-IR) at the 10-year follow-up among 524 randomly selected nondiabetic adults. Similarly, the Finnish Mobile Clinic Health Examination Survey reported an 82% lower risk of T2DM incidence in men with sufficient $25(\text{OH})\text{D}_3$ levels (mean 69.11 nmol/l) versus those with the lowest low circulating levels (mean 22.3 nmol/l, $P < .001$) after adjustment for BMI, physical activity, education,

and smoking [339]. However, a statistically significant reduced risk was not observed for women in the study, therefore, more investigations need to be undertaken for the role of baseline VitD on predicting diabetes outcomes in women.

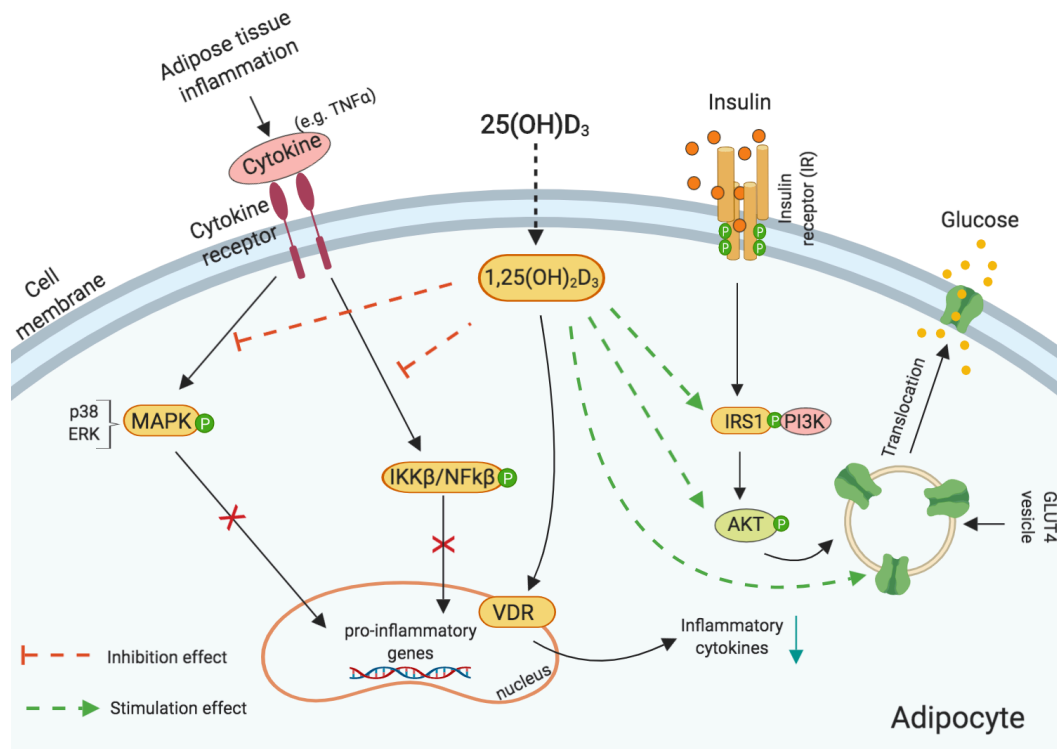


Figure 1.3.1 Suggested mechanisms of the anti-inflammatory effects of VitD and its role in glucose homeostasis: Proposed mechanism of $1,25(\text{OH})_2\text{D}_3$ signalling in adipocytes. $1,25(\text{OH})_2\text{D}_3$ may be pass into the adipocyte (from the circulation or adjacent cells) or $25(\text{OH})\text{D}_3$ taken up from the circulation and hydroxylated in the fat cell by VitD metabolizing enzymes [305]. $1,25(\text{OH})_2\text{D}_3$ acts to inhibit the phosphorylation of MAPKs and the activation of $\text{NF}\kappa\text{B}$ signalling, in human adipocytes. As a result, gene expression and protein release of proinflammatory mediators by fat cells are reduced, leading to decreased overall inflammation within adipose tissue. $1,25(\text{OH})_2\text{D}_3$ may also stimulate the phosphorylation of IRS-1 and AKT and promote GLUT4 translocation to cell membrane. (original figure drawn)

1.4 Effectiveness of current obesity treatments on obesity mediated diabetes

Health care professionals should evaluate each patient's readiness to achieve weight loss and jointly determine weight loss goals and intervention schemes in order to promote sustainable weight loss. Approaches involve diet, physical activity, behavioural therapy, pharmacological treatment, and bariatric surgery (Table 1.4.1). The latter two schemes may be advised for carefully selected patients as adjuncts to diet, physical activity, and behavioural therapy [340].

Intervention	BMI category			
	25.0 – 26.9	27.0 – 29.9	30.0 – 34.9	≥ 35
Diet and physical activity	✓	✓	✓	✓
Pharmacotherapy		✓	✓	✓
Bariatric surgery			✓	✓

1.4.1 Lifestyle intervention

Lifestyle programs that provide 1200-1500 kcal/day for women and 1500-1800 for men (or that attain a 500–750 kcal/day energy deficit) can promote weight loss, adjusted for baseline individual's body weight. Although, these amounts might only promote approximately 5% weight loss from the excess body weight, the ideal sustained weight loss is estimated to be ≥7%. Although these diets may differ in the food content and foods they restrict (such as high-fat, high-protein or high-carbohydrate diet), they have all been evidenced to be effective if the required energy restriction has been created [341-344]. The type of diet should be based on individual's health status and personal preferences. Intensive

lifestyle interventions should be a minimum of six months and include 500–750 kcal/day energy deficit diets plus physical activity. However, these interventions should be under supervision of trained interventionalists in either individual or group sessions. Overweight and obese patients diagnosed with T2DM who successfully achieved weight loss with intensive lifestyle interventions need to be enrolled in weight loss maintenance programs for at least 12 months under trained interventionalist supervision. These programs involve ongoing follow-up of body weight (weekly or more frequently monitoring), continued intake of low calorie diet, and participation in intensive physical activity (200–300 min/week) [340]. Some proprietary and commercial weight loss programs have demonstrated favourable weight loss results [345].

When provided by trained clinical practitioners, short-term intensive weight loss programs (3-months) which constitute of very-low calorie diet (less than 800 kcal/day) or total meal replacements, may promote greater weight loss (10-15%) than intensive lifestyle interventions which only promote 5% weight loss. However, weight regain has been found to be greater after the termination of very-low calorie diet than intensive lifestyle interventions unless very close follow-up sessions and weight loss maintenance programs were provided [346, 347].

1.4.2 Pharmacotherapy

Health care providers should first consider their choice of glucose-lowering medications, when evaluating pharmacological treatments for

overweight and obese patients diagnosed with T2DM. Whenever possible, medications should be chosen in a way that promote weight loss or are weight neutral. Drugs associated with weight loss include metformin, glucagon-like peptide 1 agonists, sodium–glucose cotransporter 2 inhibitors, α -glucosidase inhibitors, and amylin mimetics. The dipeptidyl peptidase 4 inhibitors (DPP-4) seem to be weight neutral. Unlike these medications, insulin, thiazolidinediones and insulin secretagogues, have often been linked with weight gain [340].

The U.S. Food and Drug Administration (FDA) has permitted five weight loss agents (or combined agents) for long-term use (longer than a few weeks) by individuals with BMI ≥ 27 kg/m² with one or more obesity associated comorbidities (*e.g.*, T2DM, dyslipidaemia, and hypertension) and by individuals with BMI ≥ 30 Kg/m² who are encouraged to lose weight [348]. These drugs include lipase inhibitor, selective serotonin (5-HT) 5-HT_{2C} receptor agonist, sympathomimetic amine anorectic/antiepileptic combination, opioid antagonist/aminoketone antidepressant combination and acylated human glucagon-like peptide 1 receptor agonist. Weight loss medications assist patients to more constantly adhere to low-calorie diets and to reinforce behavioural lifestyle changes including physical activity. Clinical health care providers should be knowledgeable about the medication label and should balance the potential benefits of successful weight loss against the potential side effects of the medication for each patient. These medications are not allowed for women who are or may become pregnant [340].

Safety and efficacy should be assessed at least every month for the first 3-months of treatment. The medication should be discontinued and alternative medications or treatment approaches should be considered if the patient did not achieve reasonable weight loss (5%) or if there are any tolerably or safety side effects at any time [340]. Generally, pharmacological treatment of obesity has been limited by modest efficacy, low adherence, weight regain after medication cessation, and adverse effects [348].

1.4.3 Bariatric surgery the “metabolic surgery”

Several bariatric procedures promote significant and long term improvement of T2DM. Given the amount of weight loss and the rapid effect of the operation on improving hyperglycaemia, as well as the experimental evidence showing the direct effect of the surgery on glucose haemostasis, bariatric surgeries have been suggested as a treatment option for T2DM and are termed “metabolic surgery” [340].

A significant body of evidence has now accrued, including data from several randomized controlled trials, showing that metabolic surgery promotes greater glycaemic control and decrease of cardiovascular risk factors in obese patients with T2DM compared with different lifestyle/medical interventions [349]. In addition, observational cohort studies have suggested that bariatric surgeries may reduce longer-term mortality [350].

According to this accumulating evidence, several organizations and authority agencies have suggested expanding the indications for bariatric

procedure to include patients with uncontrolled T2DM and BMI as low as 30 Kg/m² [340]. Longitudinal follow-up studies ranging from 1 to 5 years have shown that 30-63% of patients with T2DM prior to the surgery were in sustained remission post-operatively [351]. Available data suggest that 30-50% of patients who are in remission post-surgery experience a recurrence after approximately 8.3 years. However, with or without diabetes relapse, it is estimated that majority of patients may maintain significant improvements in glycaemic control for around 15 years post-operatively [340]. Several independent factors been suggested to predict diabetes remission and recurrence including: shorter duration of diabetes, non-use of insulin, younger age, and better glycaemic control [350, 352, 353]. Larger preoperative visceral fat area can also independently predict better postoperative outcomes, particularly among Asian American patients with T2DM, who normally have more visceral fat compared with matching Caucasians with diabetes of the same BMI [354].

In addition to improving glycaemia, randomized controlled trials have documented additional benefits that bariatric surgeries may exert, including: greater decreases in cardiovascular disease risk factors [351] and improvements in quality of life [353]. Over the past two decades, the safety of bariatric operations has dramatically improved, with continued modification of minimally invasive procedures (laparoscopic sleeve gastrectomy), involvement of multidisciplinary teams and enhanced training and credentialing. With bariatric “metabolic” surgeries, the mortality rates could be as low as 0.1–0.5%, similar to hysterectomy or cholecystectomy [355-357]. Morbidity rates have also

significantly dropped for laparoscopic procedures with a probability of 2-6% for major complications and up to 15% for minor ones [355-362]. Retrospective modelling and analyses studies suggest that bariatric operations may be cost-effective or even cost-saving for patients with T2DM, although the outcomes are largely dependent on assumptions about the safety of the procedures and long-term effectiveness [351, 363].

1.5 Pre and post bariatric surgery VitD status and its link with surgical outcomes

1.5.1 Preoperative correction of micronutrient deficiencies

Several bariatric surgeries were introduced in the past four decades, ranging from primarily restrictive, to combined restrictive/malabsorptive, to purely malabsorptive procedures. Roux-en-Y gastric bypass (RYGB) is considered as a restrictive/malabsorptive procedure where a small stomach pouch is created and connected to the bottom part of the small intestine. The biliopancreatic diversion (BPD) is a malabsorptive in which 80% of the stomach is removed. The majority of the intestine is then bypassed by connecting the end portion of the intestine to the duodenum near the stomach. The Laparoscopic Adjustable Gastric Banding (LAGB) is a purely restrictive procedure in which an inflatable silicone device is placed around the top portion of the stomach to restrict food intake. Other restrictive procedures include the Sleeve Gastrectomy (SG) where 80% of the stomach is removed leaving a tube like structure [364].

Preoperatively, operating physicians are most commonly focussed on management of co-morbidities associated with obesity such as lipid abnormalities,

T2DM, insulin resistance, cardiovascular disease, hypertension, and sleep apnoea. From a nutritional angle, surgeons often focus on caloric restriction to prepare patients for postoperative changes and neglect other micronutrient deficiencies and their associated comorbidities. Current studies have demonstrated that bariatric surgery candidates are potentially at higher risk for micronutrient deficiencies such as VitD, vitamin B12, folate, etc. However, it is still not clear whether these deficiencies would independently predict any post-surgical complications. Although there are a few studies which document a higher risk for postoperative complications for patients with pre-existing malnutrition, the effect of VitD deficiency on post-operative complications has not been adequately studied to date [365]. Given that there is a relationship between post-operative complications and micronutrient deficiency, potential bariatric candidates are routinely tested for their micronutrient status and are provided with necessary supplements. As it would be unethical to withhold supplementation from a deficient patient for the purpose of a clinical study, animal studies have been used to elucidate this point and continue to contribute to this area of research [365, 366]

1.5.2 Pre-operative VitD status

A large number of studies show low VitD status among bariatric surgery candidates [340] owing to several factors that been previously discussed. VitD deficiency was prevalent in 94% of obese subjects who were candidates for bariatric surgery [367] and been found to influence post-operative outcomes although this issue has not been well-studied [368]. Very few studies have

investigated the effect of preoperative VitD levels on weight loss and diabetes outcomes post bariatric surgery. A recent retrospective study on 46 patients undergoing bariatric surgery showed a significant correlation between baseline VitD levels with the existence of T2DM (OR 1.83; CI 1.13-2.97) [368]. Another report by Ljunghall *et al.* [369] designed to investigate the effect of 3-months VitD supplementation on glucose haemostasis, showed no differences in all diabetes parameters between the treatment and placebo groups. However, a significant reduction in body weight was observed for patients who were on VitD supplementation although they were not on a weight loss diet. The effect of VitD on other metabolic outcomes been investigated. For instance, Carlin *et al.* [370] reported that higher baseline VitD were able to prevent postoperative amelioration of hypertension. Moreover, Obispo *et al.* have concluded the presence of a relationship between preoperative VitD concentrations with persistence of postoperative metabolic syndrome such as dyslipidaemia and high blood pressure [368]. More studies are required on larger sample sizes to investigate the effects of baseline VitD on post bariatric surgery weight loss and diabetes outcomes.

1.5.3 Post-operative VitD status

Studies have reported varying degrees of postoperative VitD deficiency following bariatric surgeries [371]. In examining predictors of VitD deficiency following weight loss surgeries, one study showed preoperative VitD status to be a significant factor [285]. Other studies implicate the anatomy of the procedure as a predictor whether it is a malabsorptive or a restrictive. Vix *et al.* found that

patients who underwent gastric bypass had significantly lower VitD levels than those who underwent sleeve gastrectomy (20.5 nmol/l vs. 41.4 nmol/l respectively) [371]. However, without VitD supplementation, it seems likely that bariatric surgery contributes to post-operative VitD status and the likelihood of deficiency [285]. Furthermore, weight loss surgeries may further complicate attempts to achieve normal VitD levels. Given the nature of VitD absorption within the small intestine, malabsorptive surgeries such as the biliopancreatic diversion can make the maintenance of VitD more challenging [248]. Even with the gastric bypass (RYGB) procedure, there have been challenges to promote adequate VitD levels, given that variable characteristics such as limb length (the middle part of the small intestine) might affect absorption [372].

Summary

Obesity is characterised as a chronic inflammatory state in which adipose tissue could be a source of inflammatory mediators leading to metabolic disorders including insulin resistance and T2DM. Low serum levels of VitD (25(OH)D) is one of the most common nutrient deficiencies during obesity. Several mechanisms have been suggested that might account for low VitD status including impaired VitD metabolism within adipose tissue, whilst VitD deficiency is associated with the increased incidence of obesity and T2DM. Numerous reports have also demonstrated the role of VitD in adipose tissue metabolism and its ability to inhibit preadipocytes differentiation and to improve insulin sensitivity; although this is not fully understood. Furthermore, baseline VitD deficiency appears to contribute to different metabolic outcomes post-bariatric surgery which is

currently used as a treatment option for obesity-mediated metabolic diseases. The restrictive operation sleeve gastrectomy (SG) is currently one of the most recommended surgical options as it has better weight loss outcomes than gastric band and lower complication rates including nutrient deficiencies than the malabsorptive procedure laparoscopic gastric bypass (RYGB). However, the effect of baseline physiological VitD levels as a predictor of T2DM remission following sleeve gastrectomy has not been previously studied. Moreover, several studies have demonstrated improvements in VitD levels post major weight loss by very low calorie diet or bariatric surgeries [220, 373-375]. Thus, it is important to investigate whether these improvements are accompanied with enhancement in local VitD metabolism in adipose tissue. Hence, this thesis will aim to study three aspects:

First, the effect of baseline VitD levels on weight loss and diabetes outcomes post-sleeve gastrectomy.

Second, to investigate whether bariatric surgery-induced weight loss could result in improvements in VitD metabolic components and therefore enhancement in 25(OH)D circulating levels.

Finally, to understand the mechanistic pathways whereby VitD may exert its protective effects against inflammation and insulin resistance.

1.6 Aims and objectives

The general aim of this thesis is to address the question of the effect of VitD on obesity-mediated insulin resistance and whether VitD metabolism is affected by obesity, through investigating the influence of VitD on metabolic health and dysfunction in different metabolic states:

1. From the clinical approach, the effect of baseline serum VitD levels on weight loss and diabetes outcomes post bariatric surgery.
2. From the basic science approach:
 - a. Whether enlarged adipose tissue mass as seen in obesity might influence the level of VitD metabolic components and whether these levels can be restored with major weight loss.
 - b. The role of VitD on inflammation and glucose homeostasis in insulin resistant adipocytes.

CHAPTER 2
Materials and Methods

2.1 Ethical approval

2.1.1 Cohort study data (Audit data)

To start the audit data collection, a Clinical Audit Proposal Form that has been signed by the clinical audit lead was completed and sent to the Quality and Patient Safety under the Quality and Effectiveness Department to be able to start data collection. Then, the clinical audit proposal was approved by the University Hospital Coventry and Warwickshire NHS Trust (UHCW) and registered on Quality and Effectiveness Department proposals database with the number 1273.

2.1.2 Human adipose tissue collection

An ethical approval for the collection of human adipose tissue and serum/plasma was issued by The University Hospitals Coventry and Warwickshire NHS Trust Research and Development Department and the following approval number was provided: SK06/9309. All subjects provided informed and written consent according to the Declaration of Helsinki and had adipose tissue and bloods samples collected at University Hospitals Coventry and Warwickshire.

2.1.3 Bariatric surgery

For the bariatric study, an ethical approval was provided by The Ethics Committee of the Institute of Endocrinology, Prague, Czech Republic (EC: 19/5/2009). All subjects provided informed and written consent according to the Declaration of Helsinki and had different bariatric surgeries at the OB Clinic, Prague, Czech Republic.

2.2 Subjects

2.2.1 Cohort study subjects (audited data)

A nonrandomized, prospective cohort study was conducted on 309 morbidly obese patients undergoing sleeve gastrectomy (SG) at Warwickshire Institute for the Study of Diabetes, Endocrinology, and Metabolism (WISDEM), University Hospitals Coventry and Warwickshire NHS Trust (UHCW) between 2010 and 2018. A multidisciplinary team consisted of a bariatric surgeon, an endocrinologist, a registered dietitian and a psychiatrist evaluated all patients before and after SG. All patients were evaluated preoperatively in 4 scheduled visits and after the surgery at 3, 6, 12, and 18 months. Time between first visit and the surgery was usually between 9 and 18 months. During the preoperative period, a structured dietary intervention was conducted under the supervision of a registered dietitian which consisted of 2 parts. In the first part, nutrition advice was given to patients to modify their eating patterns, encountering a minimum goal of 5% excess body weight loss prior to surgery (this is used as an indicator of adherence to dietary and lifestyle advice - a crucial factor for long-term weight loss maintenance). During this period, all patients were scheduled for dietary counselling sessions on a regular basis to prepare them for post-surgical dietary restrictions. In the second part of the dietary intervention, patients were asked to follow a liver shrinking diet (a very low calorie diet ~800 kcal/day) 2 weeks prior to surgery as it makes it easier for the liver to move during surgery. In the early postoperative period, progressive diet adaptation instructions were reinforced to help patients maintain dietary changes consistent with surgery.

Appointments by the multidisciplinary team included anthropometric measurements (weight and height), laboratory tests, blood pressure measurements, complications and clinical assessment of any comorbidities to adjust the treatment and to detect any improvement or remission. The percentage of EWL (%EWL) was calculated based on the excess weight of that corresponding to a BMI of 25 Kg/m² for each subject. Serum variables were recorded at baseline, 6 and 12 months post SG including serum levels of 25(OH)D, thyroxine (T4), thyroid stimulating hormone (TSH), lipids profile, liver enzymes, fasting glucose, HbA1c, and C-reactive protein (one day before and after surgery). In addition, anti-diabetic medication before and after the SG was reported. Patients with different comorbidities were identified by a formal letter from the physician, endocrinologist and the psychiatrist.

2.2.2 Adipose tissue, blood samples and anthropometrics collection

A cross-sectional study of 130 non-diabetic, lean, overweight and obese women undergoing elective surgery at UHCW was undertaken. Blood and paired abdominal (subcutaneous and omental) adipose tissue samples were collected during the surgery following 8-10 hours overnight fast. Data including medical history, smoking status, and medication were all collected. Pre-surgical body mass index (BMI) was used for all analysis and Lean was considered of less than 25.0 kg/m², overweight categorised with BMI that is over 25.0 and less than 30.0 kg/m². Obese was considered as BMI over 30.0 kg/m².

2.2.3 Bariatric surgery subjects, adipose tissue, blood and anthropometrics collection

For this observational follow up study, a female cohort of forty-four Caucasian adults with morbid obesity (BMI ≥ 35 kg/m²) and T2DM were recruited who underwent bariatric surgery: laparoscopic adjustable gastric banding (LAGB; n=14), laparoscopic sleeve gastrectomy (LSG; n=16) and biliopancreatic diversion (BPD; n=14) at the OB Clinic, Prague, Czech Republic. Abdominal subcutaneous fat samples were collected by biopsy on the day of surgery (baseline) and at six months post-operatively. For the purposes of this study all subjects were prospectively investigated at baseline and 6 months post-surgery; both anthropometry and biochemical analysis were undertaken at both visits. Participants on incretin mimetics and/or insulin for T2DM treatment were excluded from this study [376]. Anthropometric measurements and plasma samples were collected at the time of surgery following 8-10 hours overnight fast and 6 months post-surgery following 8-10 hours overnight fast.

2.3 Tissue culture

2.3.1 Primary human adipocytes proliferation and differentiation

Human white primary adipocytes were derived from subcutaneous adipose tissue of six lean females (mean BMI=22.6 \pm 1.4 kg/m²) and six obese females (mean BMI=33.4 \pm 1.2 kg/m²). Cells were grown in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 – Gibco, #11039) supplemented with 10% FBS (Biowest, #S1810), 1% Pen Strep (Penicillin Streptomycin – Gibco, #15140), 1% L-Glutamine 200mM (Gibco, #25030081), and 5mg/mL Transferrin

(Fisher Scientific, #VX0030124SA). A T-75 flask was used for growing cells until they were 90% confluent, then cells were trypsinised and seeded into the appropriate plates for further experiments at density 5×10^4 cells/cm². Cells were incubated at 37°C in an atmosphere of 95% air and 5% CO₂ until they were 100% confluent. Adipocyte differentiation was initiated after 2 days of 100% confluency and was induced by incubating cells with a differentiation medium that composed of DMEM/F12, 3.5mg/ml glucose, 1% Pen Strep, 3% FBS, 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1.7 µM insulin (Sigma lot#i9278), 10 µM rosiglitazone (Cayman lot#71740), 2 nM T3 (Triiodothyronine - Merck, #T2877), 3.3 mM biotin (Sigma-Aldrich, #B-4639) and 1.7 mM pantothenic acid (Sigma-Aldrich, #P-5155). The medium was renewed every other day. At least 80% of the cells were differentiated into mature adipocytes (day 14).

2.3.2 SGBS adipocytes proliferation and differentiation

The SGBS cell lines was generated by culture of human preadipocytes originally isolated from subcutaneous adipose tissue specimen of a diseased male infant with Simpson-Golabi-Behmel syndrome (SGBS) [377]. SGBS preadipocytes are neither transformed nor immortalized, yet are able to proliferate for many generations (up to 50 passages) and retain their capacity for adipocyte differentiation as opposed to human primary preadipocytes which lose their ability to differentiate when multiplied *in vitro* [378]. The cells are efficiently differentiated in the absence of serum and albumin [377]. SGBS cells were kindly provided by Professor M Wabitsch at the University of Ulm, and were cultured/differentiated by a protocol published by himself and his colleagues

[377-379]. Cells were grown in DMEM/F12 (Invitrogen, # 31330) supplemented with 10% FBS (Thermo, #10270), 1% Pen Strep (Penicillin Streptomycin – Gibco, #15140), 3.3 mM biotin (Sigma-Aldrich, #B-4639) and 1.7 mM pantothenic acid (Sigma-Aldrich, #P-5155). A T-75 flask was used for growing cells until they were 100% confluent, then cells were trypsinised and seeded into the appropriate plates for further experiments at density 5×10^4 cells/cm². SGBS differentiation was initiated after 2 days of 100% confluency. Differentiation was started (day 0) by washing cells three times with phosphate buffered saline (PBS) and then changing to a serum- and albumin-free differentiation medium (Quick-Diff medium) [DMEM/F12 supplemented with 2 μ M rosiglitazone (Cayman, #71740), 25 nM dexamethasone, 250 μ M IBMX (Sigma-Aldrich, #I-5879), 100 nM cortisol (Sigma-Aldrich, #H-0888), 0.01 mg/mL transferrin (Sigma-Aldrich, #T-2252), 0.2 nM T3 (Sigma-Aldrich, #T-6397), and 20 nM human insulin (Thermo, #12585)]. On day 4 of differentiation, medium was changed into a 3FC medium, which is composed of DMEM/F12 supplemented with 100 nM cortisol, 0.01 mg/mL transferrin, 0.2 nM T3, and 20 nM human insulin. Cells were incubated for the first four days with a Quick-Diff medium that was changed while the 3FC medium was changed every 4 days until day 12-14.

2.3.3 SGBS treatment

Treatments were performed using day 12-14 mature adipocytes. Cells were put in nine treatment groups, including: control, VitD (10 and 100 nM) dissolved in ethanol (Sigma, #D1530), TNF α (5 ng/ml of Ethanol), 100 nM insulin in high-glucose (4.5 g/l) medium (HG/Hi), TNF α (5 ng/ml) plus VitD (10 or 100 nM),

and 100 nM insulin in high-glucose (4.5 g/l) medium plus VitD (10 or 100 nM). On the first treatment day, cells were washed with PBS and changed to LGM which is serum-free, low-glucose (1 g/l) DMEM/F12 with 0.01 mg/mL transferrin for 24 hr, however, VitD (of either doses) was added to the LGM media for all those including VitD treatment then all treatments were initiated on the second day. The control and treatment groups received fresh basal media on each day of treatment. The same volume of ethanol was applied to each well, including control wells, and treatment media was filtered through a 0.22 µm filter before being applied.

2.3.4 Justifications for VitD, TNF α , high glucose, and insulin concentrations

The concentration of VitD (10 and 100 nM) was chosen according to previous studies investigating the effect of VitD on inflammation and glucose haemostasis between 0.1 to 100 nM in different cell types [147, 290, 380-384]. A concentration of TNF α (5 ng/ml) was chosen as an average of different concentrations of TNF α that been used to induce inflammation and insulin resistance in adipocytes [147, 299, 385]. The treatment to facilitate insulin resistance was chosen according to studies which have optimized insulin resistance and hyperglycaemia conditions in adipocytes using glucose concentrations as high as 4.5 g/l (25 mM) to mimic circulating levels in patients with T2DM and insulin concentration of 100 nM [385-388].

2.3.5 Collection of condition media and protein

Conditioned media (1.5 ml), was collected from six separate wells for every sample, then centrifuged at 13000 rpm for 10 min and stored at -80 C until use. To collect protein, a lysis buffer was made with 5 ml 1x radioimmunoprecipitation (RIPA) (Millipore, #2912060) with 100 µl of dissolved protease and phosphatase inhibitors [2 Complete Mini protease inhibitor cocktail tablets (Roche, #11836153001), and 8 mg sodium fluoride (NaF, Fisher Scientific) and 20 mg sodium vanadate (Na₃VO₄, Acros Organics) in 2 mL 1x RIPA]. A volume of 80 µl of the protein lysis buffer was added to each well at 4 C, cells were scraped with sterile scrapers in each well for 30 seconds, the well contents was collected and stored at -80°C.

2.3.6 RNA extraction from adipose tissue and adipocytes

For adipose tissue, about 100 mg of tissue was used for total RNA extraction. Then, 1ml Qiazol reagent (Qiagen, #79306 UK) was added and tissue was homogenized for 40 seconds using a homogenizer. For cell cultured adipocytes, 1 ml of Qiazol was added to each well of 6-well plate, scraped and harvested into a 1.5 ml Eppendorf. Subsequently, 200µl of chloroform was added to Qiazol and been mixed thoroughly for 15 seconds. Samples then were incubated at room temperature for 2-3 minutes and centrifuged at 13000 rpm at 4°C for 30 min. The resulting aqueous layer was carefully collected into 1.5 ml RNAase-free tube and 500µl of isopropanol was added to the aqueous layer then incubated at room temperature for 10 minutes. After incubation, samples were vigorously mixed and a centrifugation step was performed at 13000 rpm at 4°C

for 30 min. Next, all the supernatant was removed leaving only the RNA pellet. The RNA pellet was then washed with 75% ethanol and the sample was centrifuged again at 13000 rpm at 4°C for 30 min. Finally, all the supernatant was removed and RNA was desolved in 10-15µl free water. RNA quantification was performed using a spectrophotometer (Nanodrop ND-1000, Labtech, UK), measuring at an absorbance of 260 nm. The ratios between the two absorbance 260/280 nm and 260/230 nm were measured to give an estimate of RNA purity. A value between 1.8 and 2.1 for both ratios was accepted as suitable RNA purity for use.

2.4 Blood biochemistry and body composition analysis

Blood samples were collected in vacutainer tubes EDTA(K2E) and were centrifuged for 10 minutes at 2000 rpm. Plasma samples were aliquoted and frozen at -80°C until time of assay. All lipid measurements were determined using the Cobas 6000 (ROCHE) analyzer and Friedewald formula was used to calculate LDL cholesterol [389]. Measurements of plasma insulin levels were performed in duplicate using human insulin ELISA kit (10-1113-01; Mercodia, Uppsala, Sweden; intra-assay %CV= 3.4; inter-assay %CV= 4.5) as per manufacturer's instructions. Insulin resistance was measured using the homeostatic model assessment of insulin resistance (HOMA-IR) using to the following formula:

$$HOMA - IR = \frac{\text{fasting glucose } \left(\frac{\text{mmol}}{\text{l}}\right) \times \text{fasting insulin } \left(\frac{\text{mUI}}{\text{l}}\right)}{22.5}$$

Serum levels of LDL cholesterol were obtained using the Friedwald formula [389] as previously described [390]. Body weight was obtained to the

nearest 0.5 kg and height to the nearest 1 cm. The percentage of excess body weight loss was calculated using the following formula:

$$EWL(\%) = \frac{[Preoperative\ weight\ (kg) - Postoperative\ weight\ (kg)]}{[Preoperative\ weight\ (kg) - Ideal\ body\ weight\ (kg)]} \times 100$$

while ideal body weight been calculated using the following equation:

$$Ideal\ body\ weight\ (kg) = 25 \times [Height\ (m)]^2$$

Body fat mass was measured by bio-impedance method (Tanita TBF-300).

Plasma 25(OH)D was measured in duplicate by Elecsys electrochemiluminescence assay, Cobas modular analytics E170, (ROCHE Diagnostics, Australia; intra-assay %CV= 4.5; inter-assay %CV= 5.6) according to manufacturer's instructions. Measurements were expressed as nanomole per litre (nmol/l). VitD deficiency, insufficiency and sufficiency were defined as the following: deficiency when plasma levels are below 25 nmol/l, insufficiency when plasma levels are between 25-50 nmol/l and sufficiency when plasma levels are above 50 nmol/l [217].

2.5 Analysis of adipose tissue and tissue culture samples

2.5.1 Oil Red-O staining

Following the growth and differentiation of SGBS cells as previously described, Oil Red-O (ORO) stock solution was prepared one day prior to the assay by dissolving 0.5g of ORO powder (Sigma, UK) in 100ml isopropanol in a water bath at 56°C for 1 hr. Working ORO solution was then freshly prepared on the day of assay by diluting stock ORO with distilled water to make a 60% solution, which

was then stirred for 10 min and then filtered through Whatman No 42 filter paper. Media was aspirated from each well and cells were washed with PBS twice. Cells were then fixed with 4% formalin for 15 minutes at room temperature (RT). Excess formalin was aspirated and cells were washed with PBS twice for 5 minutes at RT, before being rinsed with 60% isopropanol. Working ORO (500µl) was added to each well and incubated at RT for 30 minutes, then removed and cells washed once with PBS. Lipid accumulation (droplets) was then viewed under a light microscope and digital photographs were taken at 20x magnification.

2.5.2 cDNA synthesis and qRT-PCR

Samples were digested with DNase I to remove potential genomic DNA contaminants (Dnase I kit, #AMP-D1 Sigma- Aldrich). Synthesis of complimentary DNA (cDNA) was performed using 1000 ng RNA per sample and a Bionline mRNA reverse transcription kit (#BIO-65026) was used according to the manufacturer's instructions. Q-RT-PCR reactions were carried out at 50 C for 2 minutes, 95 C for 10 minutes, and then 40 cycles of 95 C for 15 seconds then 60 C for 1 min. For CYP2R1 and CYP3A4 mRNA gene expression, Pre-designed gene specific Taqman probes and primers were used (Applied Biosystems, UK, CYP2R1: Hs01379776_m1, CYP3A4: Hs00604506_m1) in a reaction mix containing TaqMan universal PCR master mix (Applied Biosystems, UK). Reactions were prepared to 12.5µl volumes in duplicate in a 384-well plate. All other genes were amplified using SYBR-green master mix from Sigma-Aldrich (JumpStart™ – S4438) and the reactions were prepared at a total volume of 8µl in duplicate using a 384-Icyler from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). TaqMan reactions were

multiplexed with the housekeeping gene L19 whereas genes amplified by SYBR-green master mix were all normalized to the separately amplified housekeeping gene L19. Gene expression was calculated according to the formula:

$$\text{mRNA expression} = 2^{-\Delta\text{Ct}}, \text{ where } \Delta\text{Ct} = \text{target gene Ct} - \text{L19 Ct}$$

Data is expressed as 1 raised to the power of the difference between control ΔCt and test ΔCt ($\Delta\Delta\text{Ct}$ method). Primer pairs are listed in Table 2.5.1 below:

Gene	Forward	Reverse
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAAA
VDR	CTAGCCTCCTCTGATAGCCTCAT	AGACACTTCAGACCCAAAGGCT
CYP27A1	GTCGTTCAAGGCTATGCC	CACATTGGACCGTACTTGGC
CYP2J2	TCCTTGTGGACTTCGAGCAG	TGTCACCAAGCTCCAAGCTA
CD14	TGCCGCTGTGTAGGAAAGAA	CGCGCTCCATGGTCGATAA
CD68	GCTTTGGATTCATGCAGGACC	TGAGCCGAGAATGTCCACTG
IL-1 β	AGCTGATGGCCCTAAACAGA	AGATTCGTAGCTGGATGCCG
IL-6	TGGCAGAAAACAACCTGAACC	CCAGTGATGATTTTCACCAGGC
IL-8	GAAGGTGCAGTTTTGCCAAGG	TGGGGTGGAAAGGTTTGGAGTA
MCP1	CAGCAGCAAGTGTCCCAAAG	GAATCCTGAACCCACTTCTGCTT
NF κ β	GGGCTATAATCCTGGACTCTTGG	TTGGCGGATTAGCTCTTTTTCC
TNF- α	GCTGCACTTTGGAGTGATCG	GCACTCGGGGTTGAGAAG
IGF-1	ATCAGCAGTCTCCAACCCA	GGAGGACATGGTGTGCATCT

2.5.3 Western blot analysis

All cells were incubated in low-glucose, insulin-free medium during the last hour to recover from chronic insulin treatment. Cells were then stimulated with 20nM of insulin for 10min before they were lysed in RIPA buffer with

protease and phosphatase inhibitors. Bradford assay was used to determine protein concentrations, whereby 2 µl of protein sample was added to 1 ml of 1:4 Bradford stock reagent (Biorad, #5000006) and colorimetrically compared to known concentrations of bovine serum albumin (BSA 1 mg/ml) which were also added to 1 ml of Bradford reagent. Western blot analysis was performed using a previously described method [391]. For Western blotting, in brief, 10-30 µg of protein samples (Table 2.5.2) were loaded onto a 12% denaturing polyacrylamide gel (GeneFlow, UK), separated by electrophoresis (70 V until proteins reached the resolving gel then 120 V until proteins were separated). A semi-dry transfer method (Trans-Blot Turbo Transfer system, Biorad, # 1704150) was used to transfer the proteins to membrane filter Immobilon-P transfer membranes 0.2 µm pore size (Biorad, #1704270), blocked for 60 minutes in 0.5 % BSA in Tris Buffered Saline (TBS, Biorad, # 1706435) with 0.1% tween-20 added (TBST) and incubated in primary antibody diluted in TBST at suitable temperature and time as specified in Table 2.5.2. Equal protein loading was confirmed by examining β-actin or GAPDH protein expression. Membranes were washed three times for 10 minutes in TBST and incubated in anti-rabbit IgG (whole molecule), horseradish peroxidase antibody produced in goat, IgG fraction of antiserum, buffered aqueous solution (Sigma #A9169). Signals were detected using chemiluminescent detection system, enhanced chemiluminescence/enhanced chemiluminescence (ECL/ECL+) (GE Healthcare, #14775048), and densitometry was conducted using ImageQuant software.

Table 2.5.2 List of primary antibodies					
Protein	Loaded protein (µg)	Primary antibody			
		Lot#	Origin	Dilution	Incubation time/Temp
p-AKT	30	Cell signalling, #9271	Rabbit	1:1000	Overnight/4°C
AKT	30	Cell signalling, #9272	Rabbit	1:1000	Overnight/4°C
p-p38	20	Cell signalling, #9211	Rabbit	1:1000	Overnight/4°C
P38	20	Cell signalling, #9212	Rabbit	1:1000	Overnight/4°C
p-ERK1/2	20	Abcam, #ab128159	Rabbit	1:1000	Overnight/4°C
ERK1/2	20	Abcam, #ab17942	Rabbit	1:1000	Overnight/4°C
NFκβ	20	Cell signalling, #8242	Rabbit	1:1000	Overnight/4°C
Glut-4	10	Abcam, #ab654	Rabbit	1:5000	Overnight/4°C
β-actin	20*	Santa Cruz, #sc-4778	Rabbit	1:10000	1 hr/RT
GAPDH	10-30*	GeneTex, #GT239	Mouse	1:1000	1 hr/RT

*The same membrane of the protein of interest was used for the housekeeping proteins.

2.5.4 Glucose uptake assay

Glucose uptake was carried out as previously described [392-394] with some modifications according to cell type [395]. Cells were grown on 6 well plates (Corning), washed once with Krebs-Henseleit buffer (KHB) with 0.01 % BSA, 5 mM glucose, 10 mM Hepes with 7.4 pH (1xKHB/low glucose/BSA). Cells were then incubated for 6 hrs with the same washing buffer (1xKHB/low glucose/BSA). Subsequently, they were washed with 1xKHB/no glucose/no BSA buffer to completely remove glucose and were incubated with the same buffer for 30 min at 37°C. Insulin treated cells were incubated with 100nM insulin for a further 15 min. The assay was initiated by addition of 1 µCi/ml of [³H]-2-deoxy-D-glucose (PerkinElmer) and deoxyglucose (100 µM) for 20 min. The uptake of glucose was terminated by washing cells twice with ice cold PBS. Cells were then harvested in 400 µl 1x RIPA per well and 300 µl of the lysate was transferred to 4 ml

scintillation fluid and radioactivity (becquerels, Bq) was counted using a scintillation counter. The remaining 100 μ l cell lysate was used to quantify protein concentration and glucose uptake was expressed as Bq/mg protein.

A 5x KHB buffer was prepared by adding the following reagents separately with thorough mixing in the listed order to 900 ml distilled and autoclaved water (dH₂O). The final volume was adjusted to 1000 ml with dH₂O,

Chemical	5X (nM)	5X (g/L)
NaCL	555	32.532
KCL	23.5	1.752
MgSO ₄	10	1.204
Na ₂ HPO ₄	6	0.852

On the day before the assay, the two following buffers were prepared and filter sterilized:

1x KHB/low glucose/BSA (500 ml): 5mM glucose (450 mg), 0.01 % BSA (50 mg), 10 mM Hepes (1.1915 g), pH 7.4

1x KHB/low glucose/BSA (500 ml): no glucose, no BSA, 10 mM Hepes (1.1915 g), pH 7.4

2.5.5 Immunofluorescence (IF) assay

SGBS cells (5×10^4 cells/cm²) were seeded onto 10 g/ml fibronectin (Invitrogen)-coated glass coverslips kept in 24 well plates. After reaching confluence, the cells were differentiated into adipocytes and preincubated with 1,25(OH)₂D₃ (100 nM) for 24 hr, followed by induction with or without TNF α at 5 ng/ml or HG/HI for another 24 hr. IF was carried out with a modified protocol [381] with prewashing (once) and post-washing (twice) with PBS and 10% Saponin

(fc 0.1%), fixing of cells for 15 min at RT in 4% formaldehyde. Cells were permeabilized for 3 min with 0.1% Triton X-100 in PBS for NF κ B assay while no permeabilization was done for Glut4 IF. Nonspecific binding sites were blocked with blocking solution (Goat serum (fc 5%) (Dako, #X090710) and 10% Saponin (fc 0.1%) in PBS) for 1 hr at RT. Cells were incubated with rabbit anti-NF-kB p65 (Cell signalling, #8242, diluted 1:400) or rabbit anti-Glut4 (abcam, #ab654, diluted 1:5000) primary antibodies overnight and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, #751094, diluted 1:1000) for 1 h in blocking solution at RT in the dark. A three times washing step with PBS and 10% Saponin (fc 0.1%) was performed before and after each incubation step with antibodies. After incubation with nuclear counterstain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, #P36935) at 1 mg/ml for 5 min, coverslips were finally mounted onto glass slides using Immunomount medium. Fluorescence images were acquired by confocal microscopy at 40x magnification through an oil objective lens.

2.5.6 Soluble CD14 determination by ELISA

sCD14 levels in SGBS condition media were determined using the Quantikine ELISA human CD14 Immunoassay (R & D Systems) following the manufacturer's protocol. Sensitivity for CD14 was 125 pg/ml and the intra-assay coefficient of variation was 5.5% while the inter-assay coefficient of variation was 6.3%.

2.6 Statistical analysis

Statistical analyses were performed using the SPSS 25.0 software. Categorical data are presented as percentages and continuous variables are reported as mean \pm standard deviation (SD) unless \pm standard error (SE) of the mean is stated. Data were examined for normality according to the Shapiro-Wilks criteria and by visual inspection of QQ-plots. Non-normally distributed variables were logarithmically transformed (natural logarithm) before use in parametric analyses. Comparisons between categorical groups and percentages were calculated by Chi-Square (χ^2) and McNemar tests. For continuous variables, One-way ANOVA was used to assess differences between multiple groups for normally distributed variables (Tukey-Kramer post hoc test) while One-way non-parametric ANOVA (Kruskal-Wallis test) was used for non-parametric data and a p value was considered significant if <0.05 . Two-tailed independent samples T-test or Wilcoxon Signed-Rank Test were used to compare mean differences between two independent groups for parametric and non-parametric data respectively. Mean differences between pre and post values of same variables were determined using 2-tailed paired T-test. Bivariate Pearson correlation analyses was used to analyse correlations between parametric variables while Spearman's test was used for correlations between non-parametric variables. Simple and multiple linear regression analysis was used to assess the effects of a single or multiple factors on an independent variable. A multiple binary logistic regression analysis (forward method) was performed to predict the odds of being a case based on the values of the independent variables (predictors).

Chapter 3

VitD as an important predictor for post sleeve gastrectomy weight gain and T2DM remission

3.1 Introduction

Obesity is considered a serious public health problem affecting more than 650 million adults worldwide in 2016 [396]. It is known to be associated with several metabolic diseases and is responsible for 44% of incidence of T2DM, 23% of cardiovascular diseases and 41% of certain cancers [397]. Moreover, morbid obesity has been observed to reduce life expectancy by 10.1 years [398]. Bariatric surgery (BS) is considered an effective treatment option especially for morbid obesity as it achieves a relatively rapid and successful long-term weight reduction, contributes to the remission of several comorbidities [399-401] and subsequent reduction in mortality [402, 403]. The restrictive operation sleeve gastrectomy (SG) is noted to have better weight loss outcome than gastric band and lower complication rates including nutrient deficiencies than the malabsorptive procedure laparoscopic gastric bypass (RYGB) [404]. However, several studies have demonstrated prevalence of nutritional deficiencies among obese patients prior to BS [405-410]. Low serum levels of VitD (25(OH)D) is one of the most common nutrient deficiencies during obesity [405]. Several mechanisms been suggested that might account for low VitD status, including tendency of obese individuals to get less sun exposure, due to less outdoor physical activity than lean individuals, high consumption of empty calorie food that are low in VitD content [256], impaired VitD hydroxylation in adipose tissue (AT) and the most likely reason for low VitD status in obesity is that, it is largely stored in the AT during increased adiposity [411, 412].

Furthermore, VitD deficiency is known to be associated with incidence of obesity [283] and T2DM. Several reports have demonstrated the role of VitD in AT metabolism and its ability to inhibit pre-adipocyte differentiation [225, 263]. Moreover, VitD is involved in insulin sensitivity pathways and insulin secretion [413]. Therefore, subjects with obesity and VitD deficiency have an increased future risk of T2DM [338, 339, 414]. According to previous data, VitD supplementation is associated with weight loss and improved β cell function among obese patients and patients with glucose intolerance or early T2DM [197, 415].

VitD deficiency is prevalent in 94% of obese subject who are candidates for BS [367] and been observed to influence post-operative outcomes [368]. Several attempts been taken to improve BS outcomes in terms of weight loss and comorbidity remission based on monitoring pre-surgical factors such as pre-surgical weight loss and quitting smoking which were linked to favourable outcomes post-operatively [416]. Around 85% of patients diagnosed with T2DM prior to BS were in remission 2 years post-operatively [417] and duration of diabetes is noted as the strongest predictor of achieved remission after surgery compared with surgery type, C-peptide levels and age [418]. However, the duration of diabetes is not a factor that can be monitored by medical team or the patient themselves. Therefore, it is important to study pre-surgical factors that can be monitored prior to the surgery. The effect of baseline physiological VitD levels as a predictor of T2DM remission following SG is unknown. Therefore, the aim of

this study was to analyse whether preoperative VitD levels may predict weight loss and T2DM remission post-SG.

3.2 Methods

3.2.1 Subjects and study design

As mentioned in section 2.2.1, this was a nonrandomized, prospective cohort study in which 309 morbidly obese patients undergoing SG between 2010 and 2018. All patients were evaluated before and after SG by a multidisciplinary team. Data collected included anthropometric measurements (weight and height), laboratory tests, blood pressure measurements, complications and clinical assessment of any comorbidities to detect any improvement or remission. Based on the excess weight of that corresponding to a BMI of 25 Kg/m², the percentage of EWL (%EWL) was calculated for each subject. Preoperatively, all patients were given a minimum goal of 5% excess body weight loss prior to surgery. Two-weeks prior to the surgery, patients were asked to follow a liver shrinking diet as it makes it easier for the liver to move during surgery.

3.2.2 Anthropometrics, clinical data and blood biochemistry

Anthropometrics, clinical and blood biochemistry data were collected on several time points including first visit (starting point), day of the surgery (baseline) and 4 follow-up visits at 3, 6, 12, and 18 months. All serum variables were recorded at baseline, 6 and 12 months post-surgery. In addition, comorbidities and anti-diabetic medication were reported before and one year after the SG (section 2.2.1 for details).

3.2.3 Statistical analysis

Statistical analyses were performed using the SPSS 25.0 software. Categorical data are presented as percentages and continuous variables are reported as mean \pm standard deviation (SD) unless \pm standard error (SE) of the mean is stated. Data were examined for normality according to the Shapiro-Wilks criteria and by visual inspection of QQ-plots. Non-normally distributed variables were logarithmically transformed (natural logarithm) before use in parametric analyses. Comparisons between categorical groups and percentages were calculated by Chi-Square (χ^2) and McNemar tests. For continuous variables, One-way ANOVA was used to assess differences between multiple groups and 2-tailed independent samples T-test to compare mean differences between two groups. Mean differences between pre and post values of same variables were determined using 2-tailed paired T-test. Bivariate Pearson correlation analyses was used to analyse correlations between parametric variables. Simple and multiple linear regression analysis was used to assess the effects of a single or multiple factors on postoperative independent variables. A multiple binary logistic regression analysis (forward method) was performed with incidence of T2DM post-operatively as the binary outcome variable and 25(OH)D and BMI as the explanatory variables.

3.3 Results

3.3.1 Demographic and biological characteristics at baseline and post-surgery

Baseline demographics for patients who were diagnosed with T2DM and were non-T2DM are shown in Table 3.3.1. Of the total study population (n=309, 75% female), more than 47% were diagnosed with T2DM for at least 1 year prior to the surgery (Table 3.3.2). No significant differences were observed between the groups in any of the starting characteristics. In addition, a summary of comorbidities at baseline and 1 year after surgery and post-surgical complications is shown in the tables below (Table 3.3.2 and Table 3.3.3). Hypertension, T2DM and gastroesophageal reflux disease (GORD) were the top three most dominant comorbidities with significant remissions post-operatively. The majority of participants were Caucasian (89.6%) and only 2.6% were black British, 4.2% Indian and 3.6% did not respond as to their ethnicity.

Table 3.3.1 Starting demographics of non-T2DM and T2DM cohorts

	Non-T2DM n= 163		T2DM n= 146		Non- T2DM vs. T2DM
	Mean±SD	Min-Max	Mean±SD	Min-Max	p value
Age (y)	46.32±9.93	26-70	48.2±10.6	23-70	0.261
Height (m)	1.66±0.1	1.48-1.91	1.68±0.1	1.45-1.91	0.076
H.Weight (Kg)	144.59±22.86	98-216	144.21±22.9	94-225	0.884
Weight (Kg)	129.58±21.4	88-187	130.7±21.27	84-208	0.647
BMI (Kg/m ²)	52.57±6.65	36-74	51.15±7.1	39-75	0.068
IBW (Kg)	78.84±7.73	57-91	71.67±7.93	57-91	0.079
Excess Weight (Kg)	75.75±19.1	32-143	73.54±20	36-134	0.545
% Excess Weight Loss (%)	19.45±13.14	-20-63	18.58±11.92	-6-58	0.545

Data are presented as means ± standard deviation (SD). Statistical differences between non-T2DM and T2DM patients were determined by 2-tailed independent T-tests, and significance p value shown in last right column. H.weight: heaviest weight in their first appointment, BMI: body mass index, IBW: ideal body weight (calculated if BMI was 25). %Excess weight loss is the percentage of baseline excess body weight that been lost prior to the surgery (calculated from the heaviest weight).

Table 3.3.2 Prevalence of comorbidities at baseline and end of the study

	Baseline % (n)	End of study % (n)	p
Hypertension	52.8 (163)	NA	NA
T2DM	47 (163)	18 (56)	<0.001*
GORD	37 (115)	1.3 (4)	<0.001*
OSA	28 (86)	9.7 (30)	<0.001*
Depression	24 (77)	11 (36)	<0.001*
Dyslipidaemia	19 (58)	NA	NA
Arthritis	16.5 (51)	NA	NA
PCOS	10.4 (32)	NA	NA
CVD	8 (25)	NA	NA
Back problems	7 (22)	NA	NA
CKD	5 (15)	NA	NA
Hernia	5 (15)	NA	NA
COPD	3.6 (11)	NA	NA
Epilepsy	1.6 (5)	NA	NA
Fatty Liver	1.3 (4)	NA	NA

Table 3.2 shows number and percentage of comorbidities at baseline and 18months post-surgery. T2DM: type 2 diabetes mellitus, GORD: gastroesophageal reflux disease, OSA: obstructive sleep apnoea, PCOS: polycystic ovarian syndrome, CVD: cardiovascular disease, CKD: chronic kidney disease, COPD: chronic obstructive pulmonary disease. Statistical differences between subgroups of qualitative variables were obtained using chi-squared and McNemar tests. * Significant difference.

	% (n)
Death	0 (0)
Leak*	2 (6)
Residual heamatoma	0.3 (1)
Constipation	0.6 (2)

Table 3.3 shows number and percentage of complications developed post-surgery. * A gastric leak, most commonly occurring at the upper staple line near the gastroesophageal junction [419].

3.3.2 Changes in clinical and biological characteristics from baseline to 1 year post surgery

Most of the metabolic syndrome components were statistically different in non-T2DM to T2DM at either baseline, 6 months and/or 12 months post operatively (Table 3.3.4). At baseline, non-T2DM subjects had mean fasting plasma glucose (FPG) of 4.85 ± 0.67 whereas patients with T2DM had mean levels of 7.91 ± 4.39 mmol/l ($p < 0.005$). Baseline HbA1c levels were also significantly ($p < 0.005$) lower in non-T2DM compared to T2DM subjects (38.09 ± 4 vs. 58.6 ± 18.87 mmol/mol respectively). Baseline levels of high density lipoproteins (HDL) were higher and triglycerides (TG) were lower in non-T2DM participants compared to T2DM subjects (Table 3.3.4). No significant differences were determined in thyroid stimulating hormone (TSH), thyroxine (T4), C-reactive protein (CRP) at baseline and one day post-surgery, systolic and diastolic blood pressure. At 12-months post-operatively, non-T2DM subjects had mean fasting plasma glucose (FPG) of 4.59 ± 0.7 whereas patients with T2DM had mean levels of 5.84 ± 2.37 mmol/L ($p < 0.005$). At one-year post-surgery, HbA1c levels were also significantly ($p < 0.005$) lower in non-T2DM compared to T2DM subjects (34.28 ± 4

vs. 43.6 ± 12.85 mmol/mol respectively). Furthermore, levels of high density lipoproteins (HDL) were higher and triglycerides (TG) were lower in non-T2DM participants compared to T2DM subjects (Table 3.3.4) at 12-months post-surgery. Both groups exhibited significant improvements ($p < 0.05$) in several clinical and biological indicators of metabolic health between baseline, six months and one year after surgery (such as with levels of FPG, HbA1c, HDL, Cholesterol, LDL, Liver enzymes (AST and ALT), and systolic blood pressure) one year after surgery (Tables in appendices).

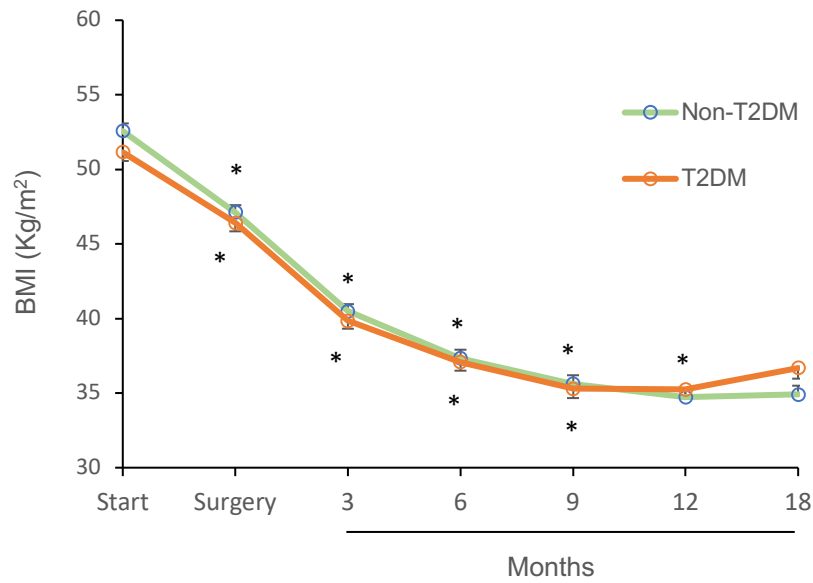
	Table 3.3.4 Comparison between biological characteristics of T2DM and non-T2DM patients at baseline, 6 and 12 months after SG														
	Baseline					6 months					12 months				
	Non-T2DM	T2DM	p value	Non-T2DM	T2DM	Non-T2DM	T2DM	p value	Non-T2DM	T2DM	Non-T2DM	T2DM	p value		
Vitamin D (nmol/l)	43.47±22.77	39.96±21	N.S	73.54±25.3	69.43±26.28	N.S	75.45±25.47	68.66±25.6	<0.05						
FPG (mmol/l)	4.85±0.67	7.91±4.39	<0.005	4.72±0.8	5.92±2.41	<0.005	4.59±0.7	5.84±2.37	<0.005						
HbA1c (mmol/mol)	38.09±4	58.59±18.87	<0.005	NA	NA	N.S	34.28±3.87	43.59±12.85	<0.005						
Cholesterol (mmol/l)	5.13±0.96	4.35±0.95	<0.005	4.89±0.95	4.3±0.95	<0.005	5.03±0.95	4.6±1	<0.005						
HDL-Chol (mmol/l)	1.33±0.32	1.15±0.33	<0.005	1.44±0.44	1.35±0.45	N.S	1.66±0.38	1.45±0.4	<0.005						
TG (mmol/l)	1.51±0.71	1.86±0.9	<0.005	1.34±0.8	1.35±0.54	N.S	1.16±0.53	1.59±0.76	<0.005						
Chol:HDL	4.02±1	4±1.25	N.S	3.61±1.1	3.39±1	N.S	3.15±0.87	3.37±1.1	N.S						
LDL-Chol (mmol/l)	3.11±0.86	2.29±0.97	<0.005	2.88±0.85	2.3±0.86	<0.005	2.86±0.89	2.45±0.95	<0.005						
AST (U/l)	77.25±21.91	80.59±25.46	<0.005	74.7±30	68.27±16.48	<0.05	70±22.54	72.89±21.23	N.S						
ALT (U/l)	23.28±13.66	28.51±18.87	<0.005	15.91±15.32	18.49±16	<0.05	13.47±5.57	15.14±7	<0.05						
CRP (mg/l)	12.1±9.7	10.88±12.8	N.S	NA	NA	N.S	NA	NA	N.S						
*CRP+1 (mg/l)	30.65±32.69	26.91±22.7	N.S	NA	NA	N.S	NA	NA	N.S						
T4 (ug/dl)	15.74±3.98	16.24±5.46	N.S	NA	NA	N.S	15.82±4.2	16.22±4.5	N.S						
TSH (mIU/l)	2.37±2	2.23±1.7	N.S	NA	NA	N.S	2.2±4.11	1.57±1.1	N.S						
Systolic BP (mmHg)	140.11±17.2	142±18.74	N.S	NA	NA	N.S	129.88±19.1	131.8±18.44	N.S						
Diastolic BP (mmHg)	76.29±10.47	76.94±10.95	N.S	NA	NA	N.S	74.65±10.41	75±10.75	N.S						

Data are presented as mean ± standard deviation of the mean. HDL: high density lipoprotein, TG: triglycerides, Chol:HDL: cholesterol ratio to HDL, LDL: low density lipoprotein, AST: aspartate transaminase, ALT: alanine transaminase. CRP: C-reactive protein, T4: thyroxine, TSH: thyroid stimulating hormone, BP: blood pressure. *CRP+1 is the level of CRP one day after the surgery. Mean differences between non-T2DM and T2DM was obtained using 2-tailed independent samples t-test.

3.3.3 Pre and post-surgical weight loss trends

Both non-T2DM and T2DM had significant BMI reduction at every follow up point (every ~3 months), though this reached a plateau at one year for non-T2DM and 9 months for T2DM cohort (Figure 3.3.1 (A)). No statistical differences between the two groups in all time points was observed. Indeed, although not statistically significant, both cohort experienced a weight regain at 18 months. Percentage of weight regained was obtained by calculating the difference between the %EWL on 18 months and 12 months divided by the %EWL at 12 months times by 100. Non-T2DM exhibited about 0.6% of weight regain at 18 months whereas 4.3% of excess weight was regained in T2DM group (Figure 3.3.1 (B)).

(A)



(B)

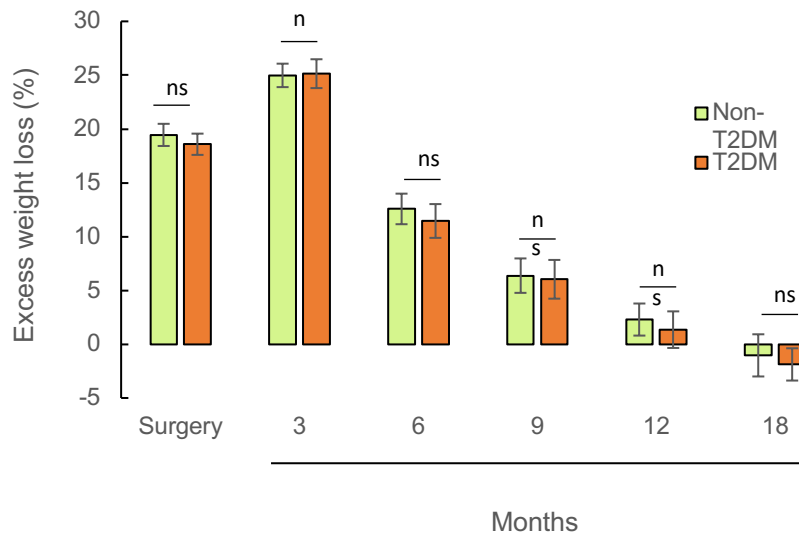


Figure 3.3.1 Trends of BMI changes and excess weight lost overtime: (A). Mean BMI throughout the service pathway from the first dietitian appointment to 18 month appointment. (B) Mean excess weight lost between every time point. Data are presented as mean \pm standard error of the mean. Differences between one time point to the next one was determined via 2-tailed paired t-test, * $p < 0.001$. Statistical mean differences between non-T2DM and T2DM were analysed using 2-tailed independent samples t-test. ns: non-significant.

3.3.4 Glycaemic changes and T2DM remission one year post operatively

Glycaemic control was evidenced by serum HbA1c levels, fasting plasma glucose levels and dose reduction in anti-diabetic treatment (Metformin). Fasting plasma glucose levels were significantly decreased at 6 and 12 months post-surgery compared with baseline levels for participants with T2DM. Interestingly, a significant reduction was interestingly observed in plasma glucose levels at one year post operatively for non-T2DM group (Figure 3.3.2 (A)). As expected, subjects with T2DM had higher HbA1c levels at baseline and one year post surgery than non-T2DM individuals, however these findings were similar to earlier glucose findings that both groups had significantly lower HbA1c levels at 12 months compared to baseline (Figure 3.3.2 (B)). Though, this reduction was significantly greater in T2DM individuals who showed an average of 23% serum HbA1c reduction compared to only 10% reduction in non-T2DM group (Figure 3.3.2 (C)).

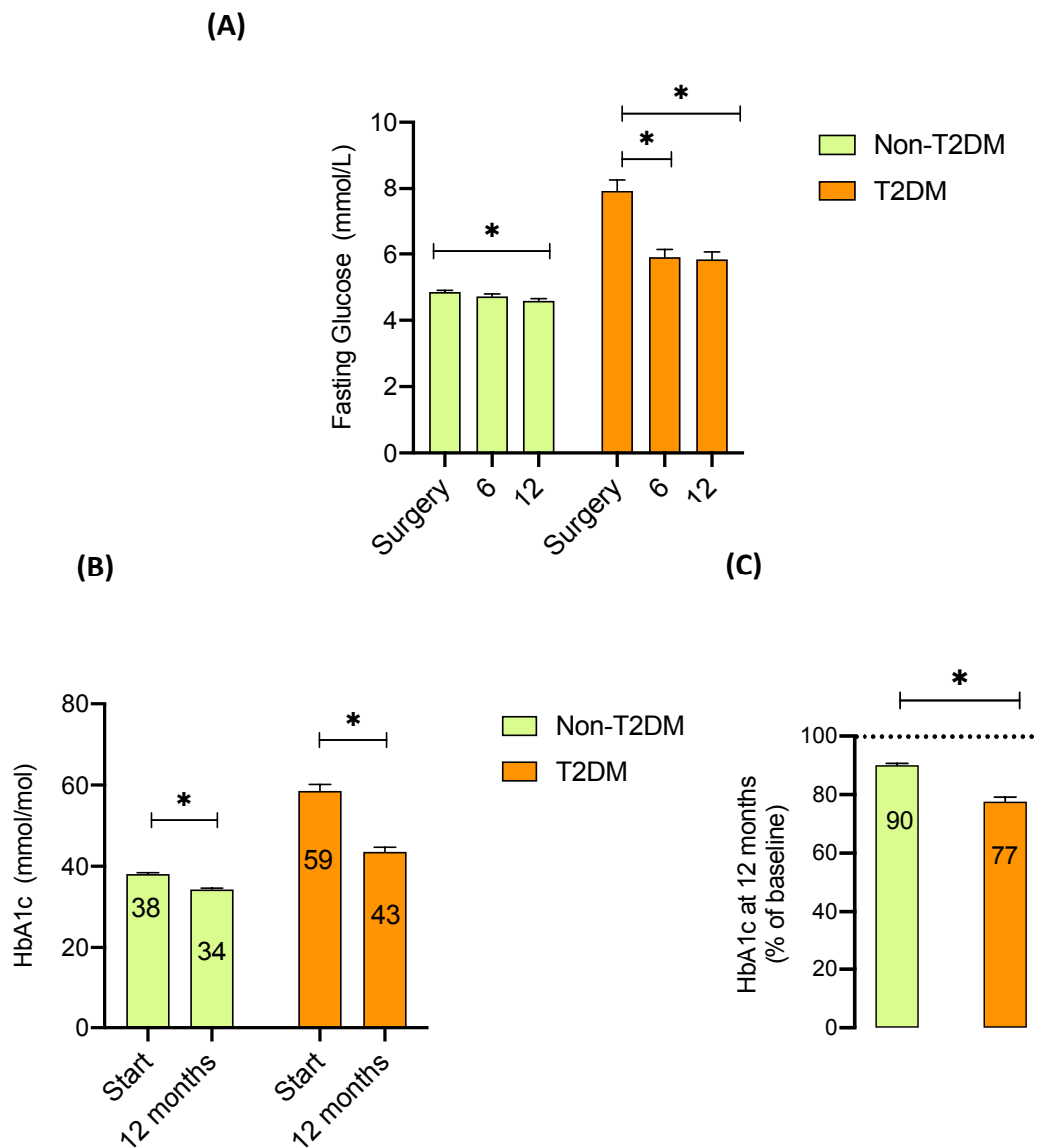


Figure 3.3.2 Achieved glycaemic control for both cohorts: (A). Fasting plasma glucose levels (FPG) at baseline, 6 and 12 months post-surgery for non-T2DM and T2DM groups. (B) Serum HbA1c levels at baseline and 12 months post-operatively. (C) Bar charts show changes in serum HbA1c levels achieved one year after surgery for both cohorts. Data are presented as means \pm standard error of the mean. Statistical differences between time points were determined via a 2-tailed paired t-test, $*p < 0.01$. Statistical differences between non-T2DM and T2DM cohorts were analysed by 2-tailed independent samples t-test.

In terms of medications, the most common prescribed anti-diabetic medication was Metformin. At baseline, more than 86% of subjects with T2DM were on Metformin treatment with an intake dose ranged between 1500mg to 250mg twice per day. A significant reduction in Metformin dosage was observed 1 year post operatively with an average intake of $260\text{mg}\pm 400\text{mg}$ (ranged between 0-1275mg twice per day) compared with $860\text{mg}\pm 300\text{mg}$ twice per day at baseline (Figure 3.3.3 (A)). According to anti-diabetic medications intake, patients with T2DM were divided into three categories according to their anti-diabetic medication intake twelve months post operatively. A group who increased or did not change the dose (Inc or N.C), a group who reduced the dose (Lower dose) and a group who ceased taking the medications (Quit). One-year post-surgery, the majority of patients with T2DM either stopped the medication (68%) or had lower dosages (12%) and only 19% increased or remained on the original doses noting that all subjects with T2DM prior to the surgery were on anti-diabetic medication (Figure 3.3.3 (B)).

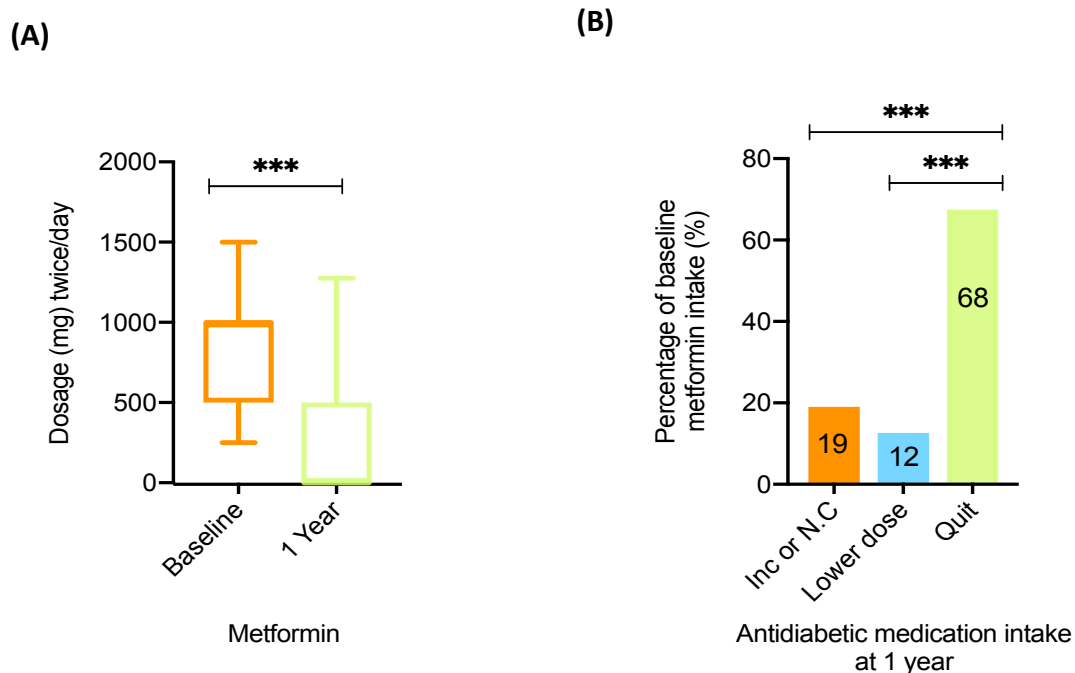


Figure 3.3.3 Changes in anti-diabetic medication intake post-surgery: (A) Boxplot displaying the distribution of Metformin intake at baseline and one year post-surgery. (B) Percentage distribution of patients who quit, lower and increased or did not change the dose 1 year post-surgery. Inc: increase the dose, N.C: no change in dose. Statistical significant differences between baseline and one year dosage were obtained via 2-tailed paired t-test whereas statistical differences between subgroups of qualitative variables were obtained using chi-squared and McNemar tests. ***p<0.001.

T2DM remission was defined as: (1) serum HbA1c levels <48mmol/mol, and fasting plasma glucose <5.5 mmol/L. (2) Absence of anti-diabetic treatments, and (3) a formal letter from the endocrinologist of T2DM remission. As previously shown in this study, about half of the patients were diagnosed with T2DM prior to the surgery. Post-surgery, over 51% of T2DM patients were in remission and only 18% of the total population remained with T2DM and out of those who remained with T2DM, 33% had lower doses of anti-diabetic medications (p<0.001) (Figure 3.3.4).

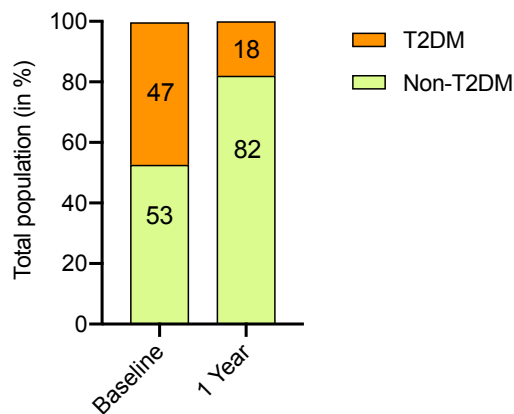


Figure 3.3.4 Percentage distribution of T2DM: Percentage distribution of T2DM in the total population at baseline and 1 year post-surgery.

3.3.5 Serum 25(OH)D levels in different ethnic groups

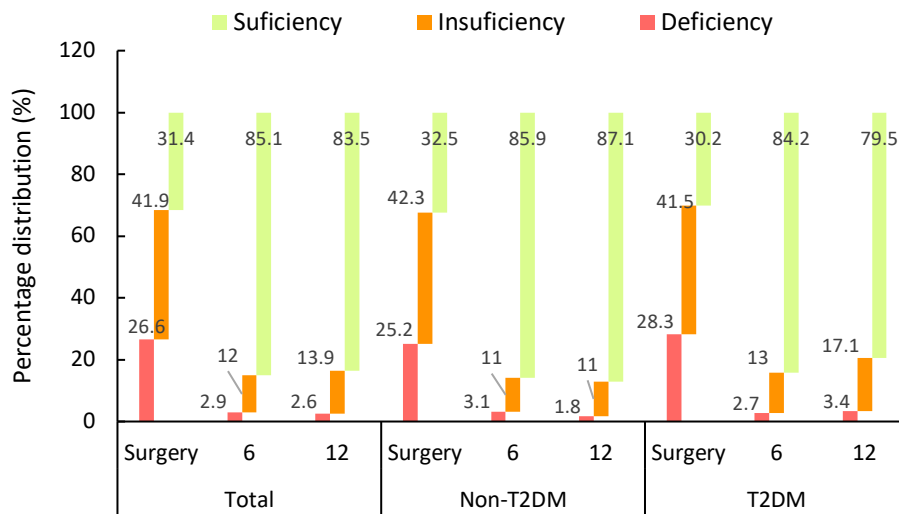
Significant variation in serum 25(OH)D levels among different ethnicities was observed ($p=0.006$). Caucasian group had significantly higher serum levels than Black British ($p=0.013$) and Indian ($p=0.012$) with mean levels of 43.2 ± 22.2 nmol/l versus 29.3 ± 21.7 and 27.7 ± 13.4 nmol/l respectively. However, the Caucasian group comprised about 90% of total population, therefore results might be affected by bias.

3.3.6 Trends of serum 25(OH)D levels from day of surgery up to 1 year post operatively

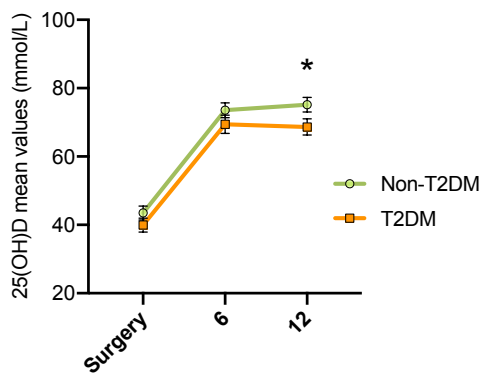
Low VitD levels (< 50 nmol/L) were prevalent in the majority of patients (69%) before undergoing the surgery with no significant differences in the mean values between both cohorts ($p = 0.170$) (Figure 3.3.5 (A-B)). After surgery, all patients were prescribed with VitD supplementation including those with normal VitD levels pre-operatively (as part of post-surgical treatment routine). This

resulted in remarkable shifts of the distribution leaving only 16% of patients who maintained low VitD serum levels. Interestingly, although both groups were on VitD supplementation, a higher percentage of low VitD was observed in T2DM than non-T2DM patients (21% vs. 13% respectively, $p < 0.05$) at one year post-surgery (mean values of 75.45 ± 25.4 nmol/l vs. 68.66 ± 25.6 nmol/l respectively, $p < 0.05$). Serum 25(OH)D was 69% and 72% higher at 6 and 12 months respectively in non-T2DM cohort and 73% and 71% at 6 and 12 months respectively in T2DM cohort (Figure 3.3.5 (C)).

(A)



(B)



(C)

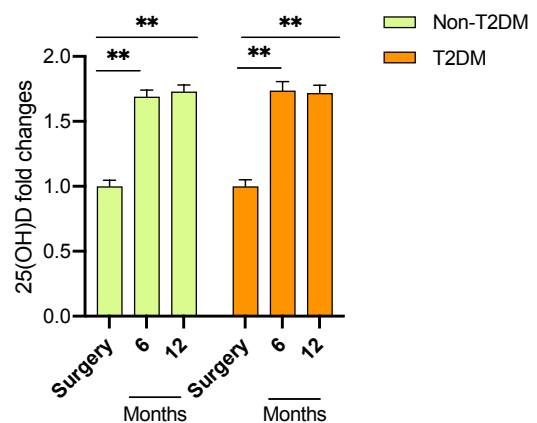


Figure 3.3.5 Summary of serum 25(OH)D improvements: (A) Bar charts showing the proportion of VitD deficiency, insufficiency and sufficiency in the combined total, non-T2DM and T2DM cohorts at time, 6 and 12 months post-surgery. (B) 2D-chart displaying mean changes in serum 25(OH)D and (C) fold changes from day of surgery to 1 year post-surgery. Error bars represent standard error of the mean. Differences between time points were determined via 2-tailed paired t-test, * $p < 0.05$, ** $p < 0.001$. Statistical differences between non-T2DM and T2DM cohorts were analysed using 2-tailed independent samples t-test.

3.3.7 Effect of VitD levels on weight loss trends post-operatively

Pearson correlation analyses were used to examine the relationship between pre-surgical VitD levels against BMI and weight loss throughout the service pathway. Pre-surgical 25(OH)D levels were significantly and negatively associated with pre and post-surgical BMI and weight loss until 9 months for weight and 6 months for BMI (coincidentally the period with fastest increase in VitD levels). However, no significant correlations were observed between baseline serum 25(OH)D and EWL at all time points (Table 3.3.5).

Table 3.3.5 Relationship of baseline serum 25(OH)D to weight and BMI throughout the service

		Baseline VitD	
		r	p value
Weight (Kg)	Starting weight	-0.237	0.0003**
	Surgery weight	-0.196	0.003**
	3m	-0.209	0.002**
	6m	-0.155	0.041*
	9m	-0.158	0.048*
	12m	-0.107	0.163
	18m	-0.149	0.069
BMI (kg/m ²)	Starting BMI	-0.254	0.0001**
	Surgery BMI	-0.190	0.004**
	3m	-0.196	0.004**
	6m	-0.193	0.011*
	9m	-0.151	0.059
	12m	-0.120	0.117
	18m	-0.147	0.147
EWL (%)	Pre-surgery EWL	0.009	0.891
	3m	0.021	0.724
	6m	0.091	0.239
	9m	0.088	0.276
	12m	0.050	0.517
	18m	0.015	0.852

Table 3.5 shows Pearson's correlation coefficient (r) and p value significance (p) between baseline serum VitD levels with weight and BMI from first appointment to 18 months post-surgery. Significant correlations are shown in red. *p<0.05, **p<0.01, n=309. BMI: Body mass index.

Baseline VitD status was categorized into three categories: VitD deficiency, VitD insufficiency and VitD sufficiency. The trend in BMI throughout the service pathway (from the start of the service pathway till 18 months post-surgery) in different categories of baseline VitD status is shown in Figure 3.3.6. The BMI of patients who were VitD deficient at baseline was significantly higher than patients with sufficient VitD levels at all time points. Patients with VitD insufficiency had also higher BMI until 9 months post operatively (not statistically significant).

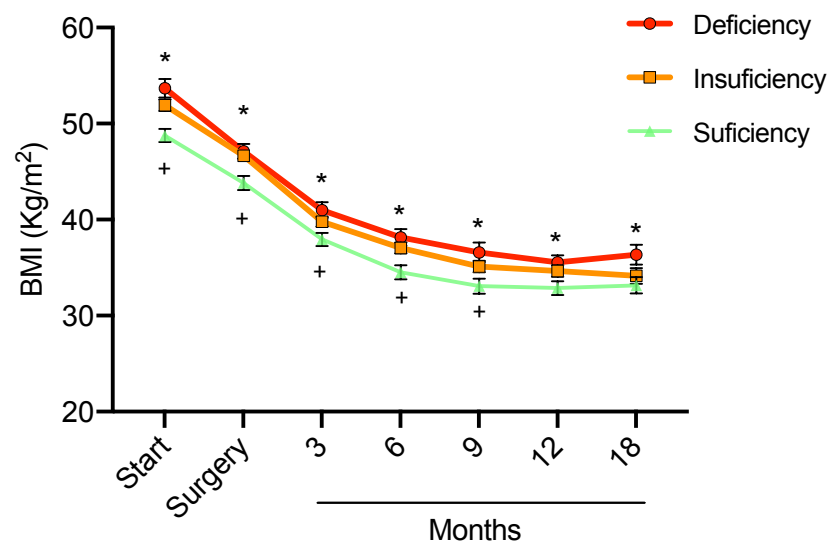


Figure 3.3.6 Trends of BMI throughout the service pathway according to VitD categories: Mean BMI trends from first appointment (Start) to 18 months post-surgery for sub-cohorts split on the basis of pre-operative VitD categories. Error bars represent standard error of the mean. Statistical differences between categories of VitD at each time point are shown as (*) for differences between deficiency and sufficiency and (+) for differences between insufficiency and sufficiency. One-way ANOVA was used for analysis (Bonferroni corrected) and a p value was considered significant if <0.05, n=229. No statistical differences were observed in BMI at all time points between patients with T2DM and non-T2DM.

Interestingly, the higher serum VitD levels at 12 months were associated with lower amounts of weight regained 18 months post-operatively among patients with T2DM who had significant lower levels of serum VitD compared to non-T2DM individuals one year post-surgery (Figure 3.3.7). Indeed, Linear

multiple regression analysis revealed that serum levels at 12 months accounted for 10% of divergence in amounts of weight regained at 18 months leaving the remaining 90% as the result of plasma glucose levels, EWL at 18 months and unknown factors (Table 3.3.6).

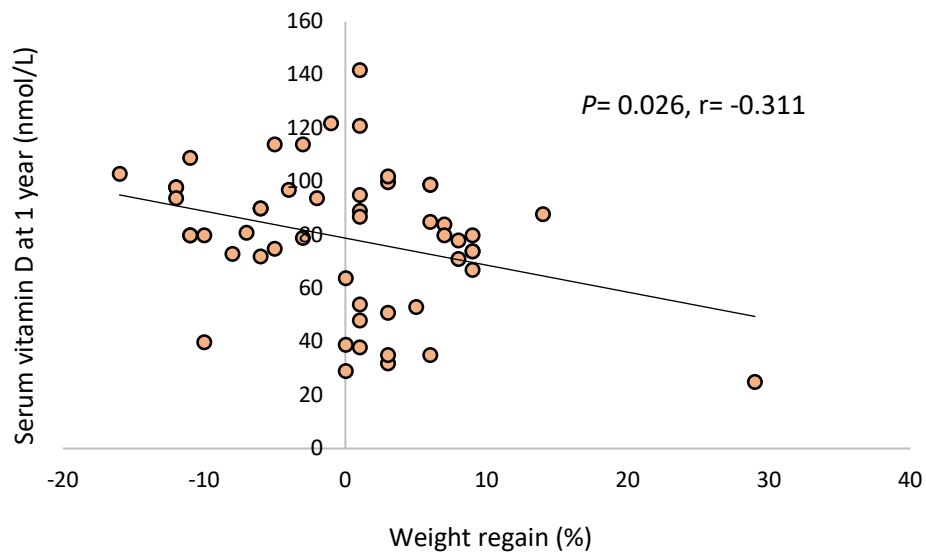


Figure 3.3.7 Association of VitD levels at 12 month with weight regain: Scatter plots showing correlation between serum VitD levels at 1 year and percentage of weight regained at 18 months post-surgery. n=57.

Table 3.3.6 Linear multiple regression model predicting weight regain at 18 months post-surgery *

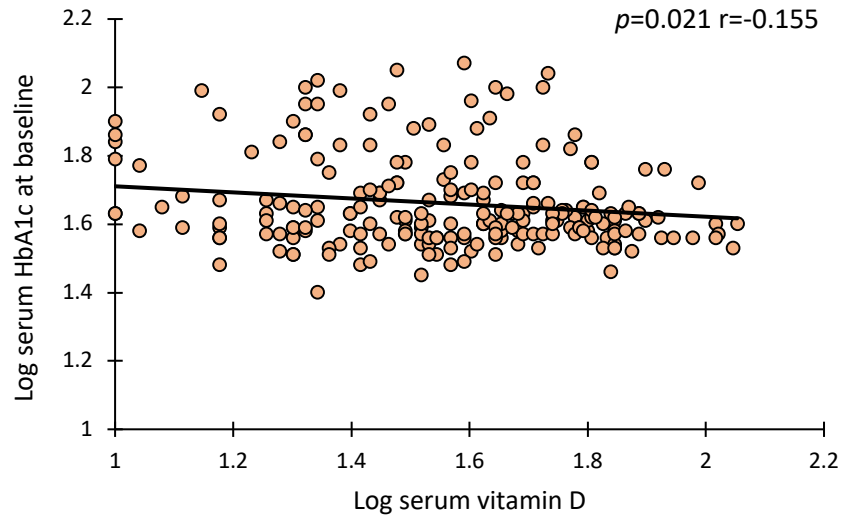
	R ² change	β Coefficient	p value
EWL - 18mo (%)	0.210	-0.458	0.001
Glucose -12m (mmol/l)	0.099	0.314	0.026
25(OH)D -12 m (nmol/L)	0.095	-0.308	0.029

A stepwise-multiple linear regression analysis was performed, using EWL at 18 months, Glucose levels and 25(OH)D at 12 months as independent predictors and weight regain as a dependent variable. *model summary p = 0.029 R² = 0.404.

3.3.8 Baseline VitD as a predictor for HbA1c levels at baseline and one year after surgery

The relationship between pre-surgical VitD levels and post-surgery T2DM outcomes was examined using Pearson correlation analyses along with linear regression analysis. A significant inverse correlation was observed between baseline VitD levels and HbA1c levels at baseline and 12 months post-surgery among total study population (Figure 3.3.8). However, this correlation was not significant in non-T2DM patients after splitting the cohort and only showed significance among patients with T2DM. Moreover, no significant relationships were determined between levels of VitD at 6 and 12 months with HbA1c levels at 12 months for total population nor after splitting.

(A)



(B)

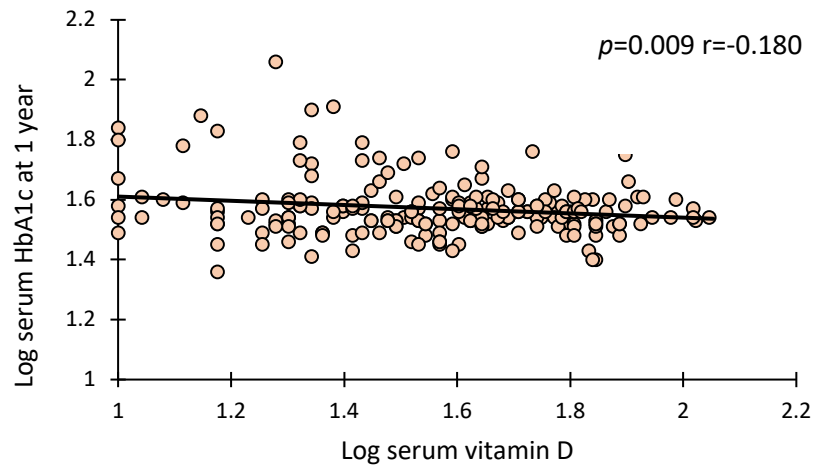


Figure 3.3.8 Association of pre-surgical VitD levels with pre/post-surgical HbA1c: Scatter plots showing correlation between pre-surgical VitD levels and (A) baseline HbA1c (n=223) and (B) HbA1c levels at 1 year post surgery (n=209). Linear trend line is shown with Pearson correlation statistic (r) and significance (p). p is considered significant if <0.05. Data were log-transformed prior to correlation analysis to improve normality.

Next, in order to ascertain whether baseline VitD can predict levels of HbA1c at 1 year post-surgery, linear regression analysis was used and revealed that baseline VitD explained 3% of HbA1c divergence (Table 3.3.7). However, after splitting the cohort based in diabetes status, the regression lost its significance in non-T2DM cohort but was stronger for patients with T2DM and showed 7.5% responsibility of serum divergence (Table 3.3.8).

Table 3.3.7 Linear multiple regression model predicting HbA1c at 1 year post-surgery for total population

	R ²	β Coefficient	p value
25(OH)D (nmol/L)	0.028	-0.180	0.009

A linear regression analysis was performed using HbA1c levels at 1 year for the total cohort as the dependent variables, and serum 25(OH)D as an independent. No other variables were entered into the model.

Table 3.3.8 Linear regression analysis predicting HbA1c at 1 year post-surgery for T2DM patients

	R ²	β Coefficient	p value
25(OH)D (nmol/L)	0.075	-0.275	0.006

A linear regression analysis was performed using HbA1c levels at 1 year for patient with T2DM as the dependent variables, and serum 25(OH)D as an independent. No other variables were entered into the model.

3.3.9 Serum 25(OH)D and HbA1c seasonal variations

If there was a seasonal variation in VitD levels and if there was a causal association between serum 25(OH)D and HbA1c then there should be also a seasonal variation for HbA1c levels. In the current study, the season where baseline 25(OH)D levels were collected was reported as it could affect the levels of VitD. However, the data on patients who were on VitD3 supplementation and who were not before the surgery were not available. Although serum 25(OH)D levels were significantly higher in autumn (September, October, November) and

winter (December, January, February) compared to spring levels (March, April, May), one-way ANOVA revealed no overall significant variations in serum 25(OH)D levels between all seasons ($p=0.111$). Similarly, no overall significant variations in seasonal baseline HbA1c levels been observed ($p=0.095$). However, there was a similarity in seasonal trends between serum 25(OH)D and HbA1c, as when VitD levels were higher in summer than spring, levels of HbA1c were lower in summer compared to spring levels and when VitD levels during autumn were significantly higher than spring, levels of HbA1c were also significantly lower in autumn compared to spring levels. Moreover, when levels of 25(OH)D were lower in winter than autumn, HbA1c levels were significantly higher in winter compared to autumn levels (Figure 3.3.9).

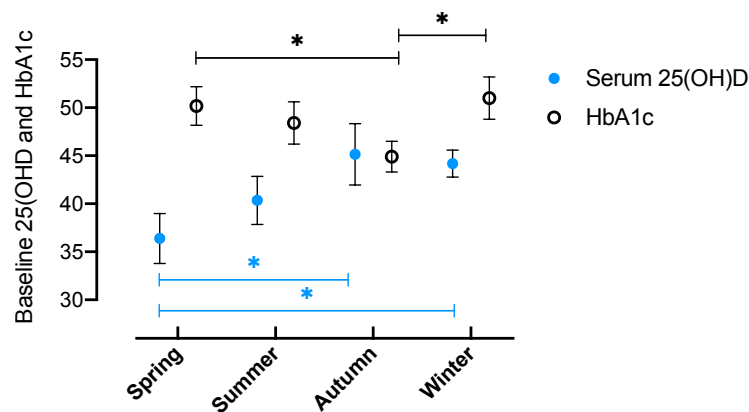


Figure 3.3.9 Seasonal variations for 25(OH)D and HbA1c: Mean \pm standard error of the mean for baseline serum 25(OH)D and HbA1c levels during the four annual seasons. Statistical seasonal variations were obtained using One-way ANOVA. Non-parametric variables were log-transformed prior to analysis to improve normality and statistical differences were computed using logged data.

3.3.10 Baseline VitD as a predictor for T2DM remission one year after surgery

As previously mentioned, intake of anti-diabetes medication at one year post-surgery was divided into three different categories: To test whether proportions of medication intake were different in each VitD category, a χ^2 test of independence was used with $\alpha = 0.05$ as criterion for significance. When patients were VitD sufficient at baseline, a higher number of patients ceased taking the medication at 1 year ($n=25$) than was expected ($n=19$), but when patients were VitD deficient, a higher number of patients remained on or increased the dose ($n=10$) than would be expected by chance ($n=5.7$). According to the χ^2 test of independence, this difference was statistically significant, $\chi^2(6, N=94) = 14.65, p = 0.023$, and results infer that patients with T2DM are more likely to quit their medication if they were VitD sufficient at baseline. In addition, one-way ANOVA was used to compare mean serum 25(OH)D values among the three different categories of medication intake one year post-surgery. A significant difference was observed in mean baseline 25(OH)D levels between patients who stopped the anti-diabetes medication and patients who increased or remained on the same dosage ($p = 0.008$) (Figure 3.3.10 (A)). No significant differences were observed when serum levels at 6 and 12 months were plotted against medication intake.

Furthermore, the link between T2DM remission and baseline VitD levels was investigated. As expected, patients with T2DM remission had significantly higher baseline 25(OH)D levels and no differences were observed in serum levels at 6 or 12 months between patients in remission and who remained with T2DM

(Figure 3.3.10 (B)). Moreover, χ^2 test of independence showed that when patients were VitD sufficient at baseline, a higher number of patients were in remission at 1 year ($n=27$) than was expected ($n=18.2$), but when patients were VitD deficient, a higher number of patients remained with T2DM ($n=20$) than would be expected by chance ($n=11.4$). According to the χ^2 test of independence, this difference was statistically significant, $\chi^2(2, N=99) = 21.5, p < 0.001$, and results infer that patients with T2DM are more likely to be in remission if they were VitD sufficient at baseline.

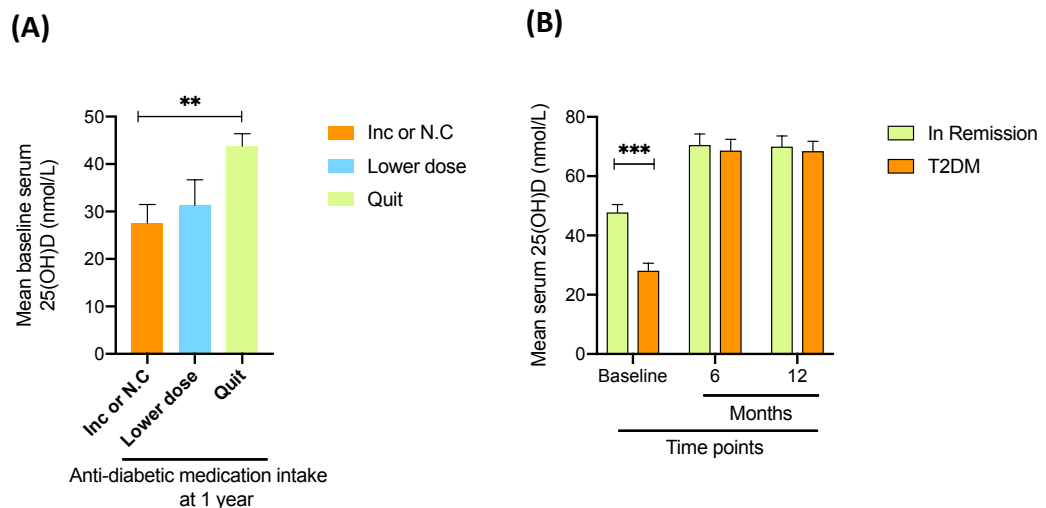


Figure 3.3.10 Mean serum 25(OH)D levels in post-surgery T2DM outcomes: Bar charts showing (A) mean serum 25(OH)D values for patients who increased or remained on the same anti-diabetes medication dosage (Inc or N.C) at 1 year post surgery ($n=19$), patients who lowered the dose ($n=10$) and patients who quit all medication ($n=65$). (B) mean serum 25(OH)D values at baseline, 6 months and 1 year post-surgery for patients who were in remission ($n=60$) and patients who remained with T2DM ($n=48$). Data are displayed as mean \pm standard error of the mean. Statistical mean differences for medication intake were determined by one-way ANOVA and statistical mean differences between the two groups of diabetes status were analysed using 2-tailed independent samples t-test. ** $p < 0.005$, *** $p < 0.001$.

Finally, in order to investigate whether serum VitD levels can predict post-surgical T2DM remission, a χ^2 test was first used to identify all comorbidities and other confounders at baseline that might be associated with presence of T2DM prior to the surgery or one year after surgery. Then, a binary logistic regression analysis was used to identify the variables which might independently predict the presence of T2DM one year after surgery. Results of χ^2 test showed that presence of T2DM at baseline was significantly associated with age, hypertension and dyslipidaemia and the presence of T2DM at one year post-surgery was associated with baseline presence of hypertension, arthritis and chronic kidney disease (CKD) (Table 3.3.9). Next, baseline serum 25(OH)D levels and the above associated variables were all included in the logistic regression model (using forward stepwise method) along with BMI and EWL (at baseline and 1 year post-surgery) as they showed significant correlations to HbA1c levels at both time points. The best fit model was obtained by a combination of baseline BMI (heaviest BMI) and serum 25(OH)D levels which together explained **33%** of the presence of T2DM one year post surgery. Serum 25(OH)D was entered the equation first ($R^2 = 0.267$) and BMI was the only other variable that played a significant role (addition to $R^2 = 0.06$). Serum 25(OH)D by itself explained **27%** of the presence of T2DM one year post operatively (Table 3.3.10).

Table 3.3.9 Relationship between age and baseline comorbidities with presence of T2DM at baseline and 12months post-surgery

	T2DM at Baseline n=	T2DM at 12m
	146	n= 56
	<i>p</i>	<i>p</i>
Age	0.005*	0.612
Ethnicity	0.680	0.566
No. of comorbidities (1-5)	1.565E-28***	0.162
Hypertension	2.3931E-9***	0.033*
Dyslipidaemia	0.000001***	0.543
Depression	0.920	0.172
PCOS	0.675	0.489
COPD	0.622	0.899
Arthritis	0.342	0.025*
CKD	0.123	0.004**
Fatty Liver	0.912	0.218
CVD	0.080	0.244
Hernia	0.123	0.397

Table 3.9 shows correlation between age and baseline comorbidities with presence of T2DM at baseline and 12months post-surgery. No. of comorbidities: the number of comorbidities a patient has (1 to 5 comorbidities). PCOS: polycystic ovarian syndrome. COPD: chronic obstructive pulmonary disease. CKD: chronic kidney disease. CVD: cardiovascular disease. Chi square (χ^2) test was used to compare subgroups of qualitative variables and to evaluate changes in the presence of comorbidities. Significant correlations are shown in red where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 3.3.10 Predictors of presence of T2DM 12 months post-surgery from a binary logistic regression

	R ²	β Coefficient	Odds ratio (95% CI)	<i>p</i>
25(OH)D (Log)	0.256	-4.97	0.935 (0.907-0.965)	<0.001
BMI (Kg/m ²)	0.06	-0.091	0.909 (0.839-0.984)	0.018

Binary logistic regression model (using forward stepwise method) was performed using diabetes status at 1 year as the dependent variables, and serum 25(OH)D and heaviest BMI as independent predictors. Serum 25(OH)D was log-transformed prior to analysis to improve normality. R²=0.316

3.3.11 Baseline VitD as an indirect factor for glucose levels improvements

To ascertain whether baseline VitD may be an indirect contributing factor to glucose levels improvements, baseline serum 25(OH)D levels were examined against clinical indicators which are known to be associated with glucose levels [420] at baseline, 6 months and 12 months post-operatively. Baseline VitD levels were associated with baseline triglycerides and with HDL levels at baseline and 6 months post-surgery (Table 3.3.11). Higher baseline serum 25(OH)D levels were associated with lower levels of baseline triglycerides levels, whereas the higher baseline serum 25(OH)D levels were found to be associated with higher levels of HDL at baseline and 6 months post operatively. Moreover, VitD at baseline predicted levels of baseline TG and HDL and explained 4% and 5% of serum levels divergence respectively (Table 3.3.12 and Table 3.3.13). Finally, there was a significant correlation between baseline TG and HDL levels with glucose levels at baseline, 6 months and 12 months post-surgery. The higher baseline TG levels was associated with higher glucose levels at all time points whereas the higher HDL levels prior to the surgery were associated with lower levels of glucose at all time points (Figure 3.3.11(A-F)).

Table 3.3.11 Relationship of baseline serum 25(OH)D to HDL and triglycerides levels

	Baseline VitD	
	r	p value
Baseline TG (mmol/L)	-0.142	0.047
Baseline HDL (mmol/L)	0.154	0.024
6-months HDL (mmol/L)	0.162	0.042

Table shows Pearson's correlation coefficient (*r*) and *p* value significance (*p*) between baseline serum VitD levels with Triglycerides levels (TG) at baseline and HDL levels at baseline and 6 months post-surgery. Only significant correlations are shown where $p < 0.05$, $n = 229$

Table 3.3.12 Linear regression analysis predicting baseline TG levels

	R ²	β Coefficient	p value
25(OH)D (nmol/L)	0.038	-0.142	0.047

A linear regression analysis was performed using HbA1c levels at 1 year for the total cohort as the dependent variables, and serum 25(OH)D as an independent. No other variables were entered into the model.

Table 3.3.13 Linear regression analysis predicting baseline HDL levels

	R ²	β Coefficient	p value
25(OH)D (nmol/L)	0.049	0.154	0.024

A linear regression analysis was performed using HbA1c levels at 1 year for the total cohort as the dependent variables, and serum 25(OH)D as an independent. No other variables were entered into the model.

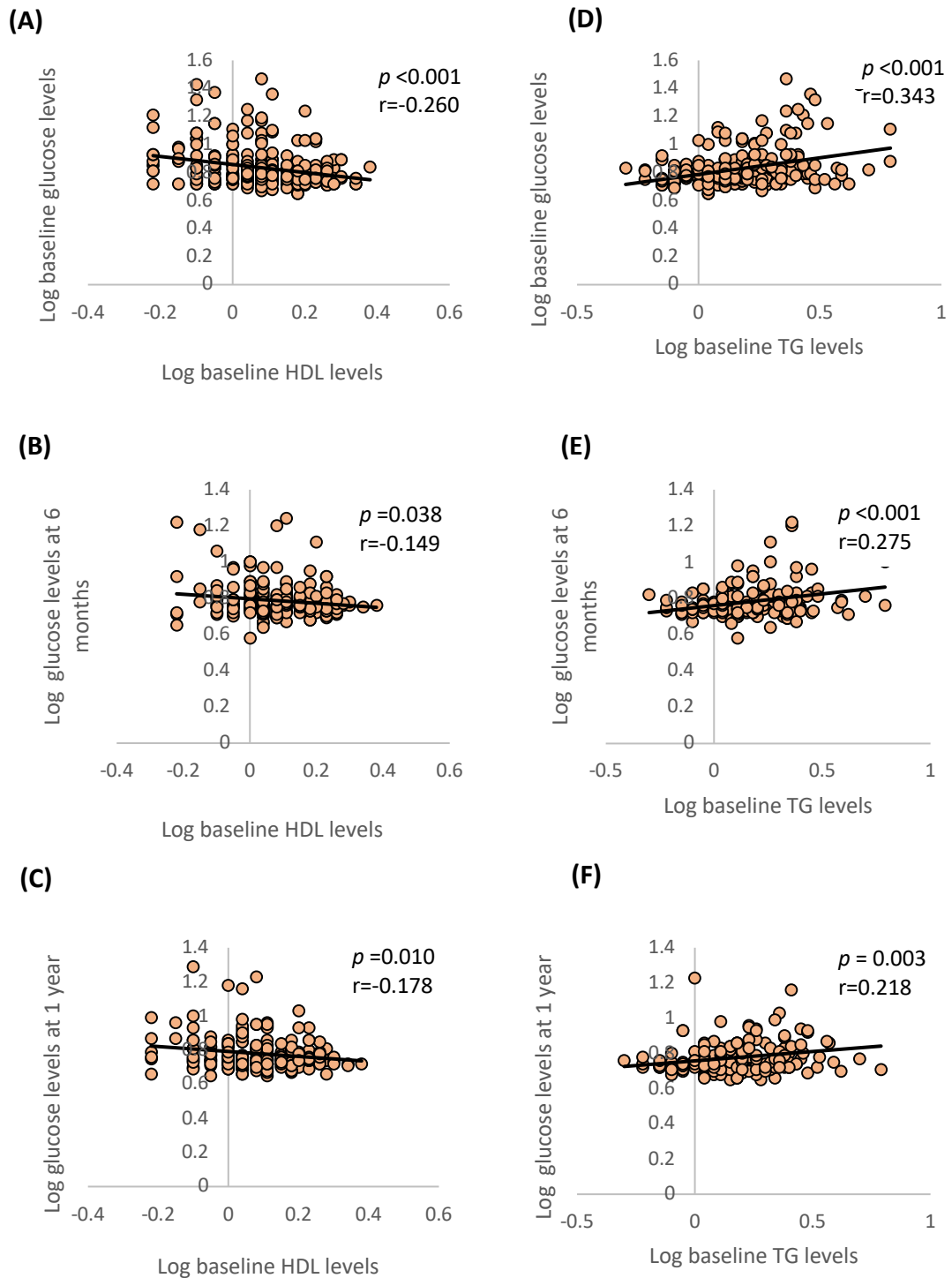


Figure 3.3.11 Association of baseline serum HDL and TG levels with Glucose levels: Scatter plots show correlations of baseline serum (A) HDL and baseline glucose levels. (B) HDL and glucose levels at 6 months post-surgery. (C) HDL and glucose levels at 1 year post-surgery. (D) TG and baseline glucose levels. (E) TG and glucose levels at 6 months post-surgery. (F) HDL and glucose levels at 1 year post-surgery. Non-parametric variables were log-transformed prior to correlation analysis to improve normality. Linear trend line is also shown with Pearson correlation statistic (r) and significance (p).

3.4 Discussion

Although there are several studies of the preoperative factors that might influence weight and diabetes outcomes post BS [421-424], the effect of baseline physiological VitD levels as a predictor of T2DM remission following BS is unknown. In this 18 month cohort study, the association of serum 25(OH)D with post-surgical weight trends and diabetes outcomes was addressed. Data analysis revealed two key findings. First: 25(OH)D might prevent weight gain but is not associated with weight loss post-operatively. Second: baseline serum 25(OH)D appears to be an important predictor for T2DM remission post SG.

Several studies have demonstrated high prevalence of VitD deficiency amongst obese patients undergoing different bariatric surgeries [425-427]. Present study shows that prior to the surgery, around 70% of patients had low serum levels of 25(OH)D with no significant differences between the sub-cohorts. Similar to these data, Fish et al, have showed that 84% of morbidly obese patients who were candidates for gastric band and bypass had low VitD levels prior to the surgery [247]. A number of mechanisms have been proposed for low levels of VitD in obese individuals including: the tendency of this group to receive less sunlight exposure [428] and sequestration of the VitD in fat tissue [284]. During long-term low VitD levels, calcium absorption is affected and the circulating levels are decreased [429, 430]. As a consequence, PTH levels will be elevated (hyperparathyroidism) in order to increase calcium levels by increasing bone turnover which eventually lead to osteoporosis [429]. Increased PTH levels were observed to be associated with weight gain as a result of reduced lipolysis [431]

and VitD supplementation was noted to suppress these levels and to prevent weight gain [432, 433]. In this study, the question whether serum 25(OH)D is associated with weight loss and regain post BS was investigated. For this baseline circulating 25(OH)D levels were first plotted against EWL, weight and BMI at all time points. Findings in this study showed that baseline circulating 25(OH)D levels were negatively associated with weight and BMI up to 9 months post-surgery. However, no correlations were observed between VitD at any time point with the amount of weight lost pre or post operatively (EWL) which align with the findings by Zitterman et al., whose study showed that baseline VitD status had no effect on subsequent weight loss [50]. These results might point toward the hypothesis that high BMI would result in more VitD sequestration and hence lower levels but higher status is unlikely to decrease the BMI [256, 284]. Interestingly, the current data in this study showed that higher serum VitD levels at 12 months, as a result of post-operative VitD supplementation, led to a significant reduced amount of weight regained at the end of the study. VitD levels at 12 months accounted for 10% of divergence in amount of weight regained 18 months post-operatively among patients with T2DM who had significant lower levels of serum VitD compared to non-T2DM individuals one year post-surgery. These data are parallel with a longitudinal study which demonstrated VitD deficiency among patients who exhibited weight regain five years post BS [434]. In addition, VitD supplementation was noted to prevent bone loss and weight gain in ovariectomized female rats [435]. The concept that VitD is involved in adipogenesis by inhibiting adipose tissue differentiation due to suppressing the genes involved in preadipocyte differentiation might explain the current findings

[263, 277, 436]. Therefore, higher VitD levels are likely to cause less differentiation of pre-adipocytes to adipocytes and hence prevent weight gain.

In the present study, results showed that low levels of baseline VitD were directly associated to HbA1c and indirectly to fasting glucose levels which eventually predicted T2DM remission twelve months post operatively. Furthermore these studies highlighted that participants with higher starting VitD had significantly lower levels of HbA1c and better post-surgical diabetes outcomes, and therefore some researchers believe that 25(OH)D is involved in better glycaemic control in patients with T2DM [437-440]. These results are consistent with the findings of a previous 2-year follow-up study. This study included 280 subjects without T2DM at baseline, however at the end of the study, 45 cases of incident of T2DM were recorded. Levels of HbA1c at the follow-up end point for those who developed T2DM were significantly higher in participants who started the study as VitD deficient. This study has also failed to find a correlation between baseline VitD and follow-up glucose levels [441]. In addition, several investigators demonstrated that baseline VitD is negatively associated with HOMA-IR and anti-diabetic medication intake at different follow-up periods [338, 339, 414]. In this current study, baseline serum 25(OH)D predicted the levels of HbA1c one-year post-surgery. The inverse association between VitD and HbA1c was stronger within patients with T2DM than total population, i.e. baseline VitD explained 3% of divergence in one-year levels of HbA1c among total population whereas it explained 8% of divergence in one-year levels of HbA1c among subjects

with T2DM. This could indicate an important role for VitD when glucose homeostasis is dysregulated [442].

Another key finding of this study, is the indirect modification of glucose levels at all time points through the inverse association of baseline VitD with TG and positive relation with HDL. In the present study, baseline 25(OH)D predicted baseline levels of TG and HDL which were strongly associated with glucose levels at all time points. These results are in line with the findings of previous study which observed a strong negative association between VitD with TG and HbA1c levels. This study showed that the association between VitD and HbA1c was most pronounced in individuals with TG above the upper reference limit [442] which indicate the indirect role of VitD in glucose haemostasis through modulating lipids profile [261, 443]. Impaired lipids profile including TG and HDL among patients with impaired glucose tolerance were also reported [444] and their status was associated with VitD status [445] and disturbed levels of HDL and TG were modified by VitD supplementation [50, 446]. These findings with the findings of current study may be supported by the notion that VitD is involved in several pathways that directly or indirectly regulate glucose homeostasis and insulin signalling [147, 265, 304, 319, 324, 382, 383].

The observed correlations between baseline VitD and HbA1c levels, anti-diabetic medication intake and lipids profiles might explain the finding this current study revealed, that baseline serum 25(OH)D predicted the presence of T2DM one year post-operatively. Prior to analysis, chi-squared test was performed to identify all baseline comorbidities and other factors that might be related to the

presence of T2DM at baseline and post-surgery. Analysis revealed that age, number of comorbidities a patient had, hypertension, dyslipidaemia, arthritis, and CKD were all associated with the presence of T2DM either at baseline or at one year post-surgery. Next, baseline serum 25(OH)D levels and previous associated variables were all included in the logistic regression model (using forward stepwise method) along with BMI and EWL (at baseline and 1 year post-surgery) as they showed significant correlations to HbA1c levels at both time points. Serum 25(OH)D was identified as the strongest predictor explaining 27% of the presence of T2DM at one year. However, this model was significantly improved when the heaviest BMI was added to the model and together explained 33% of the presence of T2DM post-surgery. These findings are aligned with a previous longitudinal follow-up study that showed 40% lower risk of developing T2DM in subjects with higher baseline 25(OH)D (>70 nmol/l) [447]. Moreover, current study showed no overall significant baseline seasonal variations in either 25(OH)D levels or HbA1c makes it no longer a confounder. Unfortunately, data on patients who were on VitD supplementation and who were committed to the supplements prior to the surgery were not available although this did not affect the aim of the study. Data from present study strongly suggests that subjects with obesity and VitD deficiency have an increased future risk of developing T2DM by 33%.

This study had certain limitations. First is the retrospective approach of the study, as the data on sun light exposure, dietary, physical activity, smoking, was not readily available. In addition, it would be helpful to have had the values of serum 25(OH)D at different times point prior to the surgery in order to compare

the increase of circulating levels until the day of surgery to the increase of circulating levels post-surgery and though investigate the effect of weight loss on serum level improvements.

In conclusion, it appears that VitD might prevent weight regain but is not associated with weight loss post-operatively. In addition, baseline VitD levels appears to be an important indicator for T2DM remission, 12 months following SG. Therefore, monitoring levels of VitD prior to BS and post-operatively is important especially for patients with T2DM in order to prevent weight regain and to improve diabetes outcomes post SG.

Chapter 4

Expression of VitD metabolic components in human adipose tissue – roles in obesity and inflammation

4.1 Introduction

The importance of vitamin D is not limited only to bone health but it also plays a crucial role in modulating acute and chronic immune responses [448-450]. This notion is supported by the findings of wide tissue distribution of VitD receptor (VDR) in different organs and cell types [451, 452], with a significant body of evidence indicating that low circulating VitD levels are associated with obesity as well as different inflammatory and chronic diseases [443, 453-458]. Furthermore, adipocytes have been demonstrated to respond to VitD with membrane bound receptors, phosphatases, nuclear co-regulator proteins [459]. VDR has the capability to regulate gene transcription either positively or negatively by interacting with response elements in gene promoters [226, 227, 264] with current studies noting that VDR gene expression is raised in conditions of obesity and several inflammatory diseases [220, 460, 461]. These higher levels of VDR suggest local VitD metabolism and a compensatory mechanism in inflammatory conditions in response to VitD insufficiency [460].

Chapter 1 outlined evidence supporting that AT is considered as an endogenous source for VitD production and metabolism especially during low cutaneous production [462]. Although current findings detail the presence of VDR expression and gene transcript of vitamin D metabolising enzymes (or CYP enzymes) in subcutaneous and visceral AT, the underlying mechanism is not fully elucidated [225, 379, 461]. Importantly, the bioactivation of 25(OH)D to 1,25(OH)₂D has been demonstrated in human adipocytes. Cherlyn and colleagues suggested that higher VDR expression in mature adipocytes indicates they are

capable of taking up 25(OH)D from the circulation, converting it to 1,25(OH)₂D₃ and then releasing the biologically active hormone to the adjacent microenvironment in AT [279]. This could explain the lower circulating levels during expanded AT in obesity [412]. CYP enzymes (also known as 25-hydroxylases) are important as they control VitD metabolism from synthesis to degradation [206]. The major circulating form of VitD is known as 25-hydroxy VitD (25(OH)D) which is used as a biomarker for VitD status. The first step of 25(OH)D synthesis involves the hydroxylation of cholecalciferol by 25-hydroxylases [208-212, 463]. Next, 25(OH)D is converted by 1 α -hydroxylase to 1,25-dihydroxy-VitD (1,25(OH)₂D₃) [206] which is the potent VDR activator [463, 464].

In human, major CYP enzymes include CYP2R1, CYP27A1 and CYP2J2 which possess 25-hydroxylase activity, with CYP2R1 being the major 25-hydroxylase and most specific [208-212]. The presence of CYP enzymes (responsible for VitD metabolism) in AT suggests vitamin D has an important role in AT metabolism noting that altered expression of these enzymes may indicate an impaired VitD activation and metabolism [220]. Furthermore, local synthesis of these enzymes has been observed to be increased by inflammatory cytokines leading to an increased local synthesis of VitD in AT to modulate or inhibit inflammatory status [305, 465], although this association has not been extensively studied.

Several studies have demonstrated improvements in VitD levels post major weight loss by very low calorie diet or bariatric surgeries [220, 373-375] and these improvements are accompanied with improvements in local VitD metabolism in AT [210, 220]. VDR gene and some of the CYP enzymes (VitD

hydroxylases), have been reported to be expressed in AT, but a systematic assessment of CYP enzymes in the AT in all BMI categories (normal, overweight and obese) has not yet been investigated. Furthermore, given that VitD levels are altered with enlarged AT [220] and that AT is a target organ for the bioactive VitD (1,25(OH)₂D₃), and its metabolism [224, 466, 467], a proposed hypothesis is that CYP enzymes and VDR rises with increasing adiposity (during obesity), which would influence the levels of circulating 25OHD. A decrease in weight and fat mass post bariatric surgery could result in improvements in circulating levels which are accompanied with improvements in local expression of VitD metabolic components. Therefore the aims of this chapter were to investigate:

1. Determine mRNA gene expression levels of VDR and VitD metabolizing-enzymes and variation between subcutaneous (AbsSc) and omental (Om) AT among different weight groups;
2. Investigate whether mRNA gene expression of VDR and VitD metabolizing-enzymes in AT may contribute to differences in VitD circulating levels;
3. Define AT specific expression of VDR and VitD metabolizing-enzymes correlation with the expression of inflammatory cytokines in AbdSc and OM AT; and
4. Evaluate whether bariatric surgeries which induce major weight loss result in improvements in VitD metabolic components and therefore improvements in 25(OH)D circulating levels.

4.2 Methods

4.2.1 Subjects and study design

Study 1: Ethical approval was obtained from the University Hospital Coventry and Warwickshire (UHCW) for the collection of human samples and issued the approval number: SK06/9309. A cross-sectional study of 84 Caucasian lean, overweight and obese women undergoing elective surgery at UHCW was undertaken. All anthropometric and biochemical investigations were conducted on the day of surgery with collection of plasma and paired AbdSc and Om AT samples at time of surgery. Study 2: this was an observational prospective study approved by Institute of Endocrinology- Ethics Committee EC: 19/5/2009, Prague, Czech Republic. Forty-four morbidly obese (BMI > 35Kg/m²), Caucasian women undergoing either bilio-pancreatic diversion (BPD; n=14), laparoscopic sleeve gastrectomy (LSG; n=16), or laparoscopic adjustable gastric banding (LAGB; n=14) at the OB clinic, Prague, Czech Republic were recruited to participate in the study. Biochemical and anthropometric investigations were conducted pre-operatively (baseline) and six months post-surgery with collection of plasma/serum samples and AbdSc AT biopsies at both time points. For both studies, all participants were consented in accordance with the Declaration of Helsinki. Data including medical history, smoking status, and medication were all collected and patients with thyroid disorders, cancer or on medications known to affect VitD levels or metabolism were excluded from the studies.

4.2.2 Anthropometrics and blood biochemistry

Anthropometric measurements and plasma samples were collected at the time of surgery following 8-10 hours overnight fast and 6 months post-surgery for Study 2 following 8-10 hours overnight fast. Plasma samples were collected in vacutainer tubes EDTA(K2E), centrifuged for 10 minutes at 2000 g and aliquoted and frozen at -80°C until time of assay. All lipid measurements were determined using the Cobas 6000 (ROCHE) analyzer and Friedewald formula was used to calculate LDL cholesterol [389]. Measurements of plasma Insulin levels were performed in duplicate using human Insulin ELISA kit (10-1113-01; Mercodia, Uppsala, Sweden; intra-assay %CV= 3.4; inter-assay %CV= 4.5) as per manufacturer's instructions.

4.2.3 Plasma 25(OH)D measurement

Plasma 25(OH)D was measured in duplicate by Elecsys electrochemiluminescence assay, Cobas modular analytics E170, (ROCHE Diagnostics, Australia; intra-assay %CV= 4.5; inter-assay %CV= 5.6) according to manufacturer's instructions. Measurements were expressed as nanomole per liter (nmol/l). VitD deficiency, insufficiency and sufficiency were defined as the following: deficiency when plasma levels are below 25 nmol/l, insufficiency when plasma levels are 25-50 nmol/L and sufficiency when plasma levels are above 50 nmol/l [217].

4.2.4 Isolation of RNA and qRT-PCR

As described in more detail in methods chapter (section 2.3.6), total RNA was extracted from 100mg of frozen AbdSc and Om AT tissue using Qiazol reagent. RNA quantification was performed and cDNA synthesis was performed using Bioline mRNA reverse transcription kit according to the manufacturer's instructions. For CYP2R1 and CYP3A4 mRNA gene expressions, pre-designed gene specific Taqman probes and primers were used in a reaction mix containing TaqMan universal PCR master mix. Reactions were prepared in duplicate. All other genes were amplified using SYBR-green master mix in duplicate. Both reactions were multiplexed with the housekeeping gene L19. Calculation of delta threshold cycle (ΔC_t) values (where $\Delta C_t = C_t$ of L19 subtracted from C_t of gene of interest). Data is expressed as 1 raised to the power of the difference between control ΔC_t and test ΔC_t ($\Delta\Delta C_t$ method). Primer pairs are listed in Table 4.2.1 below:

Table 4.2.1 Human primer pairs sequences

Gene	Forward	Reverse
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAAA
VDR	CTAGCCTCCTCTGATAGCCTCAT	AGACACTTCAGACCCAAAGGCT
CYP27A1	GTTTCGTTCAAGGCTATGCC	CACATTGGACCGTACTTGGC
CYP2J2	TCCTTGTGGACTTCGAGCAG	TGTCACCAAGCTCCAAGCTA
CD14	TGCCGCTGTGTAGGAAAGAA	CGCGCTCCATGGTCGATAA
CD68	GCTTTGGATTCATGCAGGACC	TGAGCCGAGAATGTCCACTG
IL-1 β	AGCTGATGGCCCTAAACAGA	AGATTCGTAGCTGGATGCCG
IL-6	TGGCAGAAAACAACCTGAACC	CCAGTGATGATTTTACCAGGC
IL-8	GAAGGTGCAGTTTTGCCAAGG	TGGGGTGGAAAGGTTTGGAGTA
MCP1	CAGCAGCAAGTGTCCCAAAG	GAATCCTGAACCCACTTCTGCTT
NF κ β	GGGCTATAATCCTGGACTCTTGG	TTGGCGATTAGCTCTTTTTCC
TNF-alpha	GCTGCACTTTGGAGTGATCG	GTCACCTCGGGGTTTCGAGAAG

4.2.5 Statistical Analysis

Statistical analyses were performed using the SPSS 25.0 software. Data are reported as mean \pm standard deviation (SD) unless \pm standard error of the mean (SE) is stated. Data were examined for normality according to the Shapiro-Wilks criteria and by visual inspection of QQ-plots. Comparisons between paired sample i.e. SC vs. OM were performed via Wilcoxon Signed-Rank Test. A P value of 0.05 was considered as significant. For multiple comparisons (e.g. between BMI groups) differences were assessed using One-way non-parametric ANOVA (Kruskal-Wallis test) and a *p* value was considered significant if <0.05 . Spearman's test was used for correlations between non-parametric variables. Simple and multiple linear regression analysis were used to test for independent variables. A pooled reference sample was used in order to be able to compare between different PCR plates. For Pearson correlation analyses, change variables were calculated as $[(\text{post/pre}) \times 100]$ and were log-transformed prior to analysis if non-parametric.

4.3 Results

4.3.1 Demographic and biochemical data

Clinical, anthropometric and biochemical characteristics of the study group are shown in Table 4.3.1. Subjects were divided into lean, overweight and obese. Mean insulin levels were highest among obese individuals (lean: 39.8 ± 28.3 pmol/l, overweight: 65.3 ± 39 pmol/l, obese: 84.5 ± 53.6 pmol/l, $p < 0.001$). HDL levels, however, were the highest among lean subjects and lowest among obese individuals (lean: 1.7 ± 0.4 mmol/l, overweight: 1.6 ± 0.3 mmol/l, obese: 1.5 ± 0.4 mmol/l, $p = 0.013$). Overall VitD were significantly different between the groups ($p = 0.034$) in which lean showed the highest levels, however, there was no significant differences in VitD levels between overweight and obese subjects (lean: 49.1 ± 22.2 nmol/l, overweight: 36.88 ± 14.4 nmol/l, obese: 40.1 ± 26.6 nmol/l). No other statistical differences were observed in clinical, anthropometric and biochemical characteristics between the three weight groups.

Table 4.3.1 Demographic and biochemical data of all subjects

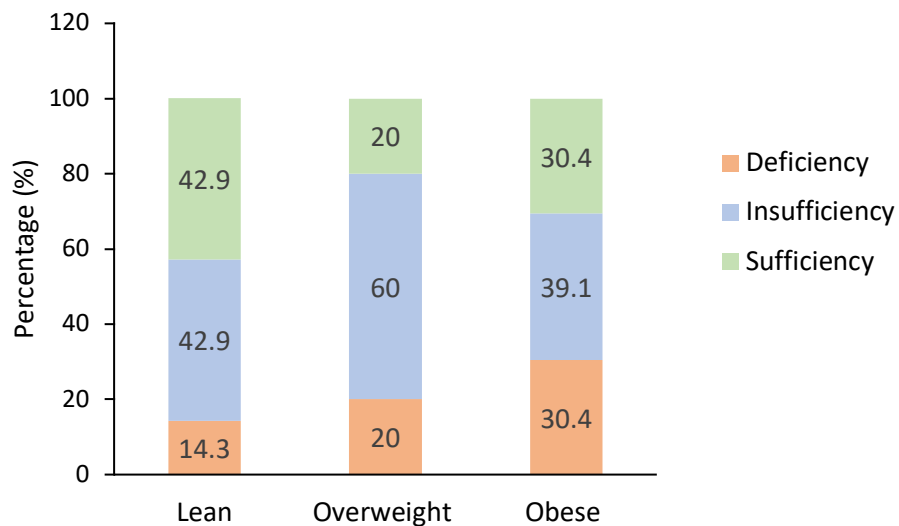
	Lean n= 29			Overweight n= 31			Obese n= 24			p value
	Mean±SD	Min-Max	Mean±SD	Min-Max	Mean±SD	Min-Max	Mean±SD	Min-Max		
Age (y)	32±5.4	22-41	31.5±7	20-59	31.2±5.8	18-43	18-43	0.799		
Height (m)	1.63±0.05	1.48-1.75	1.63±0.07	1.52-1.86	1.64±0.05	1.52-1.78	1.52-1.78	0.598		
Weight (Kg)*	59±6.3	45-72	73.4±8.6	60-95	95.6±14	74-136	74-136	<0.001		
BMI (Kg/m ²)*	22.16 ± 1.9	17.6-24.9	27.4±1.3	25-29.8	35.2±4.7	30-47.7	30-47.7	<0.001		
ADP (ng/ml)	75.6±40.8	19.2-183.2	70±30.3	27-144.5	69±48.4	23-309.2	23-309.2	0.725		
Glucose (mmol/l)	3.6±0.6	2.4-5.4	3.6±0.6	2.7-5.5	3.8±0.8	2.7-5.9	2.7-5.9	0.180		
Insulin (pmol/l)*	39.8±28.3	4.2-109	65.3±39	15.5-185.3	84.5±53.6	27.5-277.2	27.5-277.2	<0.001		
CRP (mg/l)	4.2±3.6	0.5-20.7	4.6±3.5	0.6-14.2	6.7±9	0.03-54	0.03-54	0.107		
Cholesterol (mmol/l)	6.9±1.4	4.4-10.2	6.7±1.3	3.6-10.6	6.3±1.4	2.2-9.8	2.2-9.8	0.124		
HDL (mmol/l)*	1.7±0.4	1.13-2.7	1.6±0.3	0.7-2.7	1.5±0.4	0.9-2.3	0.9-2.3	0.013		
LDL (mmol/l)	4.5±1.2	2.2-7.6	4.5±1.3	1.3-8.8	4.1±1.2	1.3-7.1	1.3-7.1	0.313		
TG (mmol/l)	3.1±1	1.1-6.4	3.2±0.9	1.6-5.8	3.1±1.2	0.3-6.3	0.3-6.3	0.865		
Vitamin D (nmol/l)*	49.1±22.2	16.6-86.4	36.88±14.4	10.5-67.8	40.1±26.6	7.5-123	7.5-123	0.034		

Data are means ± standard deviation. Comparisons between the groups were determined using One-way ANOVA using Bonferroni correction for multiple comparisons. P value was considered significant if $p < 0.05$. BMI: body mass index, ADP: Adiponectin, CRP: C-reactive protein, ALT: Alanine transaminase, ALP: alkaline phosphatase, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TG: triglycerides. All plasma measurements corresponded to fasting status.

* indicate a significant difference between the weight groups where the p value is < 0.05 .

4.3.2 Plasma 25(OH)D and adipose tissue VDR gene expression as affected by body weight

The current data highlights that 21.5 % of subjects were VitD deficient, 47% were categorized under 25(OH)D insufficient groups and 31.5% of participants were VitD sufficient. Although it did not reach a statistical difference, VitD sufficiency was the greatest among lean subjects whereas obese group had the highest percentage of VitD deficiency (Figure 4.3.1).



Plasma 25(OH)D (nmol/l)	
mean±SD	42±22
median	37.28
mode	7.5
min-max	7.5-123.1

Figure 4.3.1 25(OH)D categories distribution in different weight groups: Bar charts depicting 25(OH)D deficiency, insufficiency and sufficiency percentage distribution in 29 lean, 31 overweight and 24 obese women. Descriptive statistics for 25(OH)D levels are shown below the chart.

As aligned with previous findings, an inverse correlation was observed between BMI and plasma 25(OH)D, as BMI increases VitD levels decline ($p = 0.007$, $r = -0.244$) (Figure 4.3.2 (A)). Moreover, these data showed that BMI positively associated with VDR mRNA gene expression of AbdSc and Om VDR gene expression (Figure 4.3.2 (B-C)).

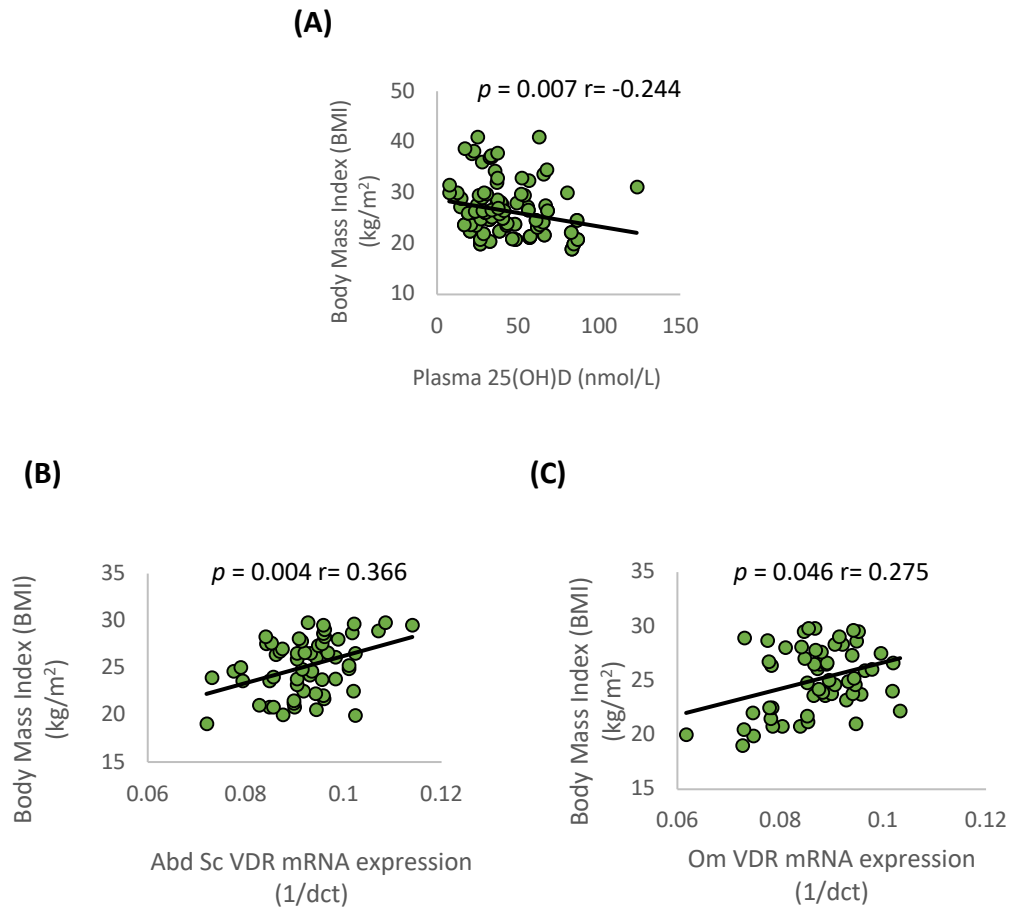


Figure 4.3.2 Association of BMI with plasma 25(OH)D levels and VDR expression: Scatter plot showing (A) inverse correlation between plasma 25(OH)D and BMI. (B) positive correlation between BMI and AbdSc VDR mRNA (C) positive correlation between BMI and Om VDR mRNA. Linear trend line is shown with correlation statistic (r) and significance (p) shown in each plot.

4.3.3 Depot-specific expression of VitD metabolic components

To study the depot-specific expression of VDR and VitD hydroxylases, the relative abundance of VitD metabolic components mRNA in AbdSc and Om AT was determined by RT-PCR and expression values in AbdSc were set at one to assess fold change from this value in Om AT gene expression. VDR and all VitD metabolizing enzymes were expressed in AbdSc and Om AT with depot specific manner for each gene. Expression of VDR mRNA was higher in AbdSc compared to its expression in Om AT among all weight groups (Figure 4.3.3 (A)). VDR gene expression in lean, overweight and obese women was 6% ($p < 0.05$), 11% ($p < 0.05$) and 6% ($p < 0.05$) higher in AbdSc than in Om AT respectively. The findings from these studies also showed that CYP2R1 expression was higher in AbdSc than in Om AT in all groups (5% ($p < 0.001$), 3% ($p < 0.005$) and 3% ($p < 0.005$) higher in lean, overweight and obese AbdSc than Om AT respectively) (Figure 4.3.3 (B)). In contrast, this depot-specific expression was reversed with CYP2J2 mRNA expression, as evidenced by 33%, 52% and 28% higher expression levels in Om AT than in AbdSc in lean ($p < 0.001$), overweight ($p < 0.001$) and obese ($p < 0.001$) women respectively (Figure 4.3.3 (C)). The expression of CYP27A1 was 8% lower in lean AbdSc compared to Om AT ($p < 0.05$) with no significant differences in overweight and obese other fat depots (Figure 4.3.3 (D)).

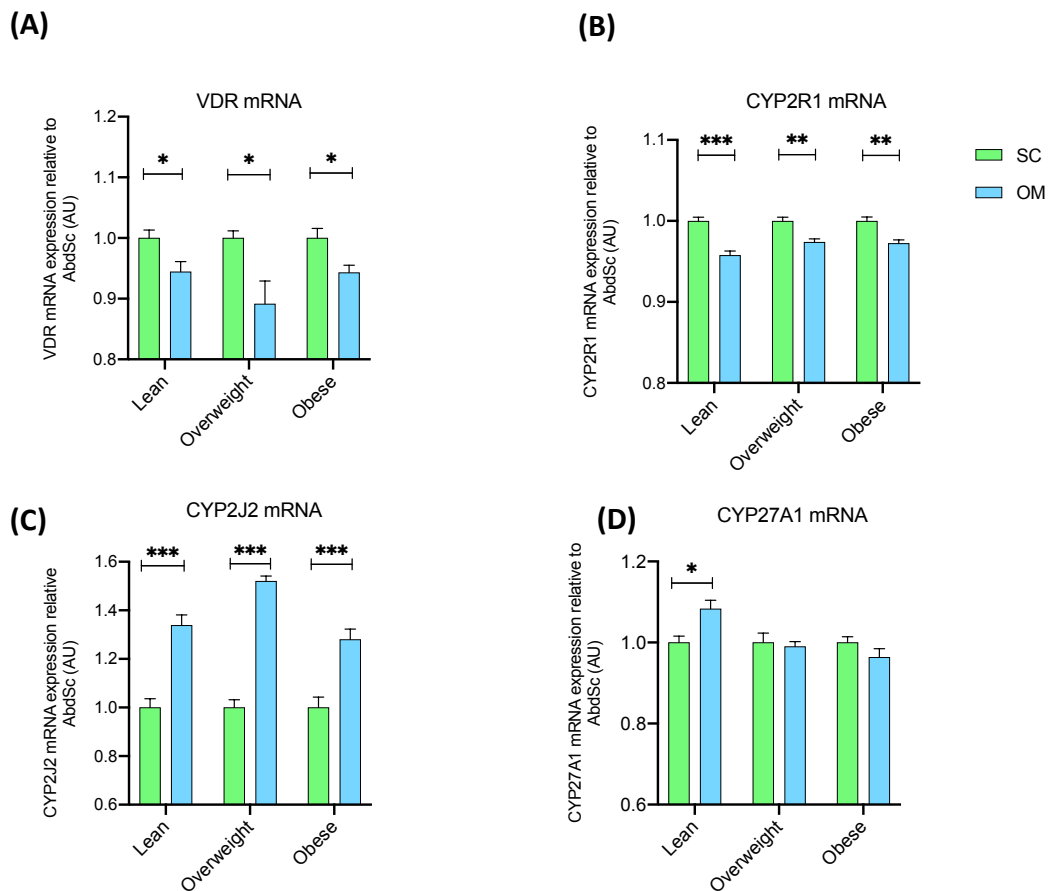


Figure 4.3.3 Depot-specific expression of VDR and VitD metabolizing: VDR and CYP enzymes gene expression were compared between AbdSc (green) and Om (blue) fat depots in 29 lean, 31 overweight and 24 obese women. Expression values in AbdSc were set at one to assess fold change from this value in Om AT gene expression. (A) VitD receptor (VDR). (B) VitD-25-hydroxylase (CYP2R1). (C) VitD-25-hydroxylase (CYP2J2). (D) VitD-25-hydroxylase (CYP27A1). Bars represent means \pm standard error of the mean. Mean differences between fat depots were determined via Wilcoxon Signed-Rank Test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$

4.3.4 Association between Plasma 25(OH)D and VitD metabolic components

To study the relative contribution of VitD metabolic components in Om versus AbdSc fat to the overall plasma 25(OH)D, the relative abundance of CYP enzyme mRNA in these two AT was determined by RT-PCR using L19 mRNA as an internal control. To evaluate this, changes in plasma 25(OH)D were examined against VDR and CYP enzymes gene expression. Plasma 25(OH)D levels were significantly correlated with AbdSc fat expression levels of VDR, CYP2R1 and

CYP27A1 mRNAs and higher expression levels of these genes were correlated with lower circulating 25(OH)D (Figure 4.3.4). In contrast, a significant direct relationship was observed between expression levels of CYP27A1 mRNA in Om AT and plasma 25(OH)D. Raised expression levels of CYP27A1 in Om AT were associated with higher plasma 25(OH)D levels.

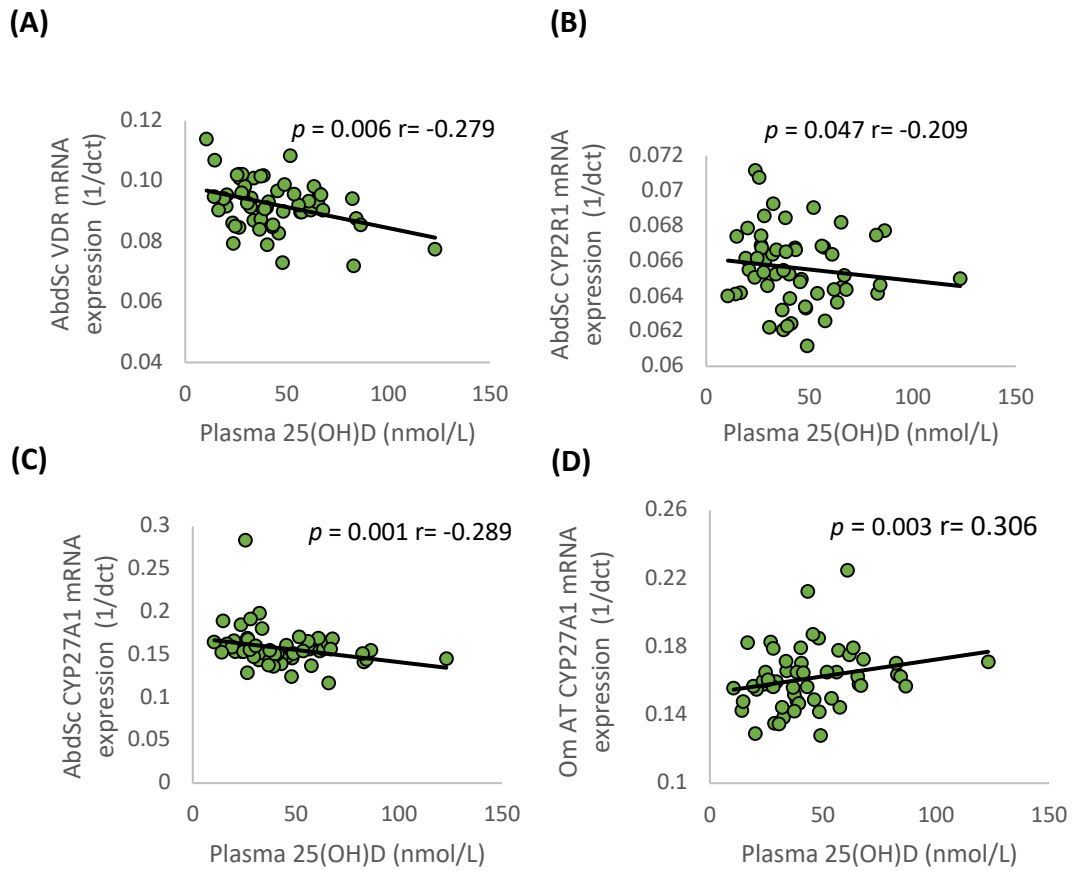


Figure 4.3.4 Correlations of plasma 25(OH)D and gene expression of VitD metabolizing enzymes: Scatter plot showing (A) inverse correlation between plasma 25(OH)D and VitD receptor (VDR) mRNA expression in AbdSc AT. (B) inverse correlation between plasma 25(OH)D and VitD-25-hydroxylase CYP2R1 in AbdSc fat depot. (C) inverse correlation between plasma 25(OH)D and VitD-25-hydroxylase CYP27A1 in AbdSc AT and (D) positive correlation between plasma 25(OH)D and VitD-25-hydroxylase CYP27A1 in Om AT. Linear trend line is shown with correlation statistic (r) and significance (p) shown in each plot.

4.3.5 VitD metabolic components in different weight groups

Expression levels of VDR mRNA were 35% ($p < 0.05$) and 17% more abundant in overweight AbdSc and Om AT respectively compared with AT from lean participants, and this expression was 32% and 21% higher in obese AbdSc and Om fat compared with its expression levels among lean participants AT respectively (Figure 4.3.5 (A)). No significant differences were observed on expression levels of CYP2R1 between the groups from both depots (Figure 4.3.5 (B)). However, obese individuals had significantly raised expression of CYP2J2 mRNA in AbdSc fat depot ($p = 0.034$) (Figure 4.3.5 (C)). Furthermore, AT expression of VitD-25-hydroxylase (CYP27A1) in AbdSc, was 37% ($p = 0.037$) and 24% ($p = 0.069$) higher in overweight and obese women respectively compared with lean subjects but it was 14% and 23% less expressed in overweight and obese individuals respectively; although this did not reach significance (Figure 4.3.5 (D)).

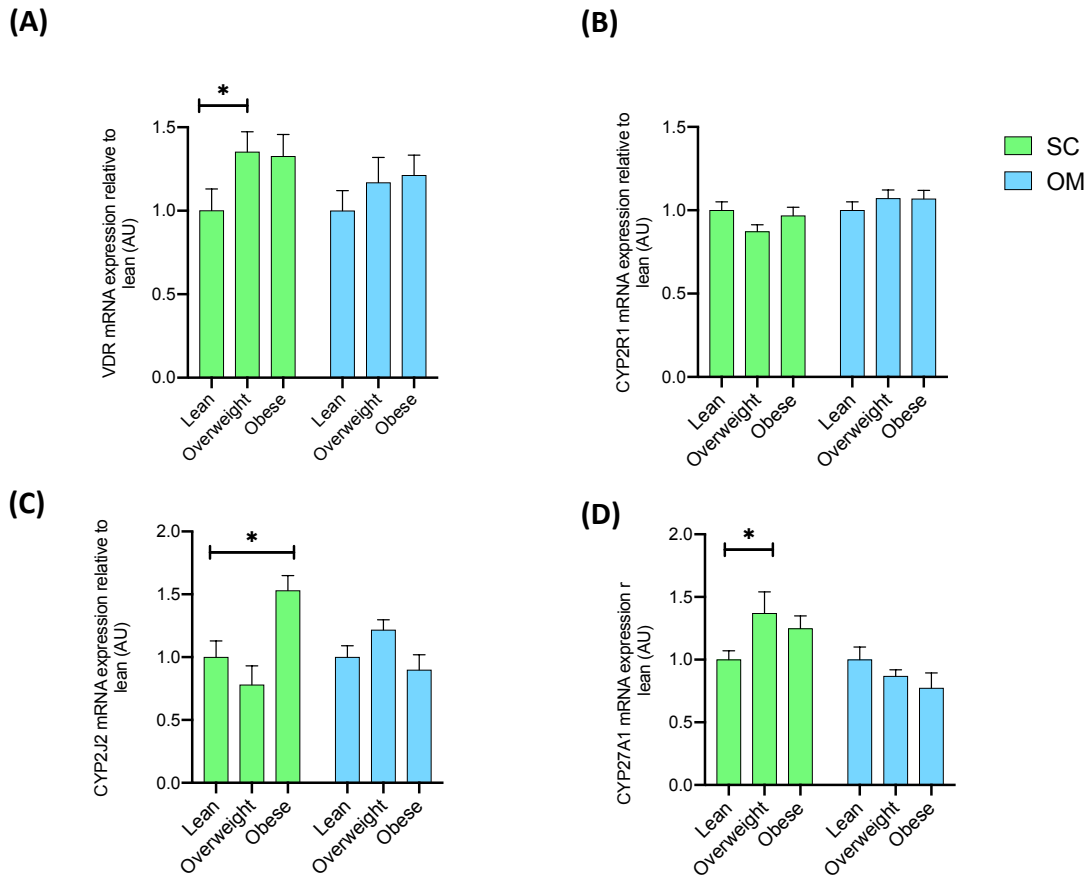


Figure 4.3.5 VDR and VitD metabolizing enzymes gene expression in adipose tissue: VDR and CYP enzymes gene expression were compared between lean, overweight and obese subjects using both AbdSc and Om samples of 29 lean, 31 overweight and 24 obese women. Bars represent means \pm standard error of the mean. A) VDR mRNA expression levels relative to Lean (control) in each AbdSc and Om AT. B) CYP2R1 mRNA expression levels relative to Lean (control) in each AbdSc and Om AT. C) CYP2J2 mRNA expression levels relative to Lean (control) in each AbdSc and Om AT. D) CYP27A1 mRNA expression levels relative to Lean (control) in each AbdSc and Om AT. Expression values in lean women were set at one to assess fold change from this value in overweight and obese women. * $p < 0.05$ One-way non-parametric ANOVA (Kruskal-Wallis test).

4.3.6 Association of VDR mRNA with clinical and inflammatory parameters expression levels

The question of whether the VDR gene is involved in systemic inflammation and metabolic health was investigated. To evaluate this, the relationship of VDR against clinical indicators of metabolic health and inflammatory cytokine expression levels were examined through Spearman's analyses. No association was observed between AbdSc VDR gene and any of the clinical variables except for HDL. Indeed, multiple linear regression analysis revealed that AbdSc VDR gene along with triglycerides were responsible for 15% of divergence in circulating HDL levels (Table 4.3.3). Furthermore, VDR levels of expression in both depots were significantly and directly associated with CRP and all inflammatory cytokines including CD14, CD68, IL-1 β , IL-6, IL-8, MCP1, NF κ B and TNF-alpha.

Table 4.3.2 Linear correlation analysis between VDR expression and clinical and inflammatory parameters

	AbdSc VDR n = 82		Om VDR n = 81	
	Spearman's rho	<i>p</i>	Spearman's rho	<i>p</i>
CRP (mg/L)	0.081	0.311	0.198	0.029
HDL (mmol/L)	-0.231	0.010	-0.111	0.281
CD14 (dct)	0.506	0.00001	0.377	0.003
CD68 (dct)	0.236	0.002	0.255	0.001
IL-1B (dct)	0.612	0.000001	0.498	0.000002
IL-6 (dct)	0.467	0.000002	0.378	0.008
IL-8 (dct)	0.350	0.000002	0.409	0.006
MCP-1 (dct)	0.542	0.000001	0.132	0.122
NF κ β (dct)	0.547	0.000001	0.384	0.005
TNF-alpha (dct)	0.404	0.00002	0.376	0.0000001

*Bivariate spearman's rho correlation is used for non-normally distributed data. AbdSc VDR mRNA expression was examined against AbdSc expression of inflammatory mediators and Om VDR mRNA expression was examined against Om expression of inflammatory mediators. CRP: C-reactive protein, HDL: high-density lipoprotein. Significant correlations are shown where p value is <0.05.

Table 4.3.3 Linear multiple regression model predicting the plasma HDL levels*

	R square change	β Coefficient	<i>p</i> value
TG (mmol/L)	0.097	-0.316	0.000002
SC VDR	0.049	-0.208	0.016
BMI	0.021	-0.136	0.092
25(OH)D (nmol/L)	0.017	0.131	0.128

A stepwise-multiple linear regression analysis was performed, BMI and 25(OH)D were included in the model but they didn't improve the prediction of the dependent variable (HDL) so been excluded by the analysis, in contrast, addition of the SC VDR to the model significantly improved the prediction. *model summary $p = 0.016$ $R^2 = 0.140$

4.3.7 Correlations between CYP enzymes, anthropometric measurements and inflammatory cytokines

In order to determine whether anthropometric factors can affect the expression levels of VitD metabolizing enzymes and whether these enzymes are associated with inflammatory genes, changes in CYP enzymes expressions were examined against anthropometrics and expression of multiple inflammatory cytokines via Spearman's analyses. AbdSc VitD-25-hydroxylases (CYP27A1) and (CYP2J2) were associated with BMI. Higher BMI was associated with higher expression of AbdSc CYP27A1 and CYP2J2. No association was observed between Om AT expression of all these enzymes with BMI. The expression of these enzymes in AbdSc and Om AT were correlated with expression levels of inflammatory cytokines. Strong parallel relationships of VitD-25-hydroxylases (CYP27A1, CYP2R1 and CYP2J2) with chemokine expression in both fat depots were observed. The higher expression of CYP27A1, CYP2R1 and CYP2J2 in AbdSc was associated with higher expression of inflammatory cytokines in AbdSc and a higher expression of CYP27A1, CYP2R1 and CYP2J2 in Om AT was associated with higher expression of inflammatory cytokines in Om AT (Table 4.3.4, Table 4.3.5 and Table 4.3.6).

Table 4.3.4 Linear correlation analysis between CYP27A1 expression with BMI and inflammatory mediators

	SC CYP27A1 n = 82		OM CYP27A1 n = 82	
	Spearman's rho	<i>p</i>	Spearman's rho	<i>p</i>
Weight (kg)	0.262	0.000002	0.081	0.128
BMI (Kg/m ²)	0.303	0.022	0.177	0.290
CD14 (dct)	0.618	0.000	0.705	0.000001
CD68 (dct)	0.539	0.007	0.421	0.000002
IL-1B (dct)	0.497	0.000	0.345	0.000004
MCP-1 (dct)	0.269	0.042	0.098	0.142
NFκβ (dct)	0.346	0.003	0.444	0.00002
TNF-alpha (dct)	0.495	0.000007	0.428	0.00001

*Bivariate spearman's rho correlation is used for non-normally distributed data. AbdSc CYP27A1 mRNA expression was examined against AbsSc expression of inflammatory mediators and Om CYP27A1 mRNA expression was examined against Om expression of inflammatory mediators. Significant correlations are shown where *p* value is <0.05.

Table 4.3.5 Linear correlation analysis between CYP2R1 expression with BMI and inflammatory mediators

	SC CYP2R1 n = 82		OM CYP2R1 n = 80	
	Spearman's rho	<i>p</i>	Spearman's rho	<i>p</i>
CD14 (dct)	0.349	0.0003	0.332	0.001
CD68 (dct)	0.192	0.027	0.219	0.091
TNF-alpha (dct)	0.187	0.253	0.410	0.000001

*Bivariate spearman's rho correlation is used for non-normally distributed data. AbdSc CYP2R1 mRNA expression was examined against AbsSc expression of inflammatory mediators and Om CYP2R1 mRNA expression was examined against Om expression of inflammatory mediators. Significant correlations are shown where *p* value is <0.05.

Table 4.3.6 Linear correlation analysis between CYP2J2 expression with BMI and inflammatory mediators

	SC CYP2J2 n = 77		OM CYP2J2 n = 81	
	Spearman's rho	<i>p</i>	Spearman's rho	<i>p</i>
CD14 (dct)	0.224	0.001	0.429	0.000001
CD68 (dct)	0.092	0.621	0.374	0.000001
IL-1B (dct)	0.189	0.021	0.495	0.00004
IL-6 (dct)	0.087	0.737	0.317	0.00001
NFκβ (dct)	0.022	0.815	0.393	0.000003
TNF-alpha (dct)	0.058	0.539	0.217	0.014

*Bivariate spearman's rho correlation is used for non-normally distributed data. AbdSc CYP2J2 mRNA expression was examined against AbsSc expression of inflammatory mediators and Om CYP2J2 mRNA expression was examined against Om expression of inflammatory mediators. Significant correlations are shown where *p* value is <0.05.

4.3.8 Association between plasma 25(OH)D with clinical parameters and chemokines

To determine whether 25(OH)D may contribute to the changes in metabolic health, changes in plasma 25(OH)D were examined against clinical indicators of metabolic health including lipid profiles and chemokine expression. Linear correlation analysis showed that circulating 25(OH)D was significantly associated with circulating HDL levels. Raised circulating 25(OH)D levels were associated with increased plasma HDL levels. Interestingly, there was a significant negative association between 25(OH)D and IL-1 β , CD68 and CD14 (Table 4.3.7).

Table 4.3.7. Linear correlation analysis between plasma 25(OH)D with clinical and inflammatory parameters

	Plasma 25(OH)D n = 126	
	Spearman's rho	<i>p</i>
HDL (mmol/L)	0.211	0.019
SC IL-1 β (dct)	-0.242	0.008
SC CD68 (dct)	-0.252	0.006
SC CD14 (dct)	-0.201	0.029

*Bivariate spearman's rho correlation is used for non-normally distributed data. Only significant correlations are indicated where *p* value is <0.05.

4.3.9 Effect of weight loss on plasma 25(OH)D levels and AT expression of VitD metabolic components

The weight loss study group consisted of forty-four morbidly obese female patients who had three different bariatric surgeries and lost a significant weight six months post-surgery. All surgical procedures resulted in significant reductions of BMI with significantly greater weight reduction in BPD group ($p=0.004$) (Table 4.3.8).

At baseline, 95% of the total population had VitD deficiency and this came down to 88% post-operatively. Among all surgeries, plasma levels of 25(OH)D were increased by 24% post-operatively although this did not reach significance ($p=0.071$). Patients who had LSG had 95% increase in plasma levels whereas patients who underwent LAGB and BPD had 27% and 25% lower plasma levels compared to baseline respectively, however, this also did not reach a level of significance. The expression of CYP27A1 was significantly decreased among all surgeries by 30% with significant differences within the surgeries ($p=0.016$). Patients who had LSG exhibited 41% decrease in CYP27A1 expression compared to baseline ($p=0.002$) whereas patients who had the BPD exhibited 9% decrease in CYP27A1 expression compared to baseline ($p=0.019$). However, no change was observed in levels of CYP27A1 with LAGB. Finally, no significant changes been demonstrated in VDR gene expression (Figure 4.3.6).

Table 4.3.8 Anthropometric, biochemical and clinical variables pre-surgery and 6-months post BPD, LGCP, and LAGB bariatric procedures

	LSG			LAGB			BPD		
	Pre	Post	Δ(%)	Pre	Post	Δ(%)	Pre	Post	Δ(%)
n	16			14			14		
Age (y)	53.2 ± 7.5			53.6 ± 11.3			50.5 ± 5.8		
EWL (%)	17.4 ± 6.3			20.2 ± 9.4			30.7 ± 8.4††		
Weight (Kg)	109.2 ± 16.5	97.6 ± 14.1**	89.5 ± 3.2	119.6 ± 19.1	105.3 ± 18.3**	88.1 ± 5.7	127.7 ± 22.6	106.9 ± 18.5**	83.8 ± 3.0
BMI (Kg/m ²)	40.41 ± 5.54	36.05 ± 4.62**	89.3 ± 3.4	43.95 ± 6.60	38.31 ± 7.05**	88.90 ± 4.9	46.45 ± 7.75	39.01 ± 6.37**	84.1 ± 3.5†
Body fat (%)	48.49 ± 3.78	44.90 ± 3.71**	92.7 ± 4.6	49.54 ± 3.44	46.39 ± 4.45*	93.6 ± 5.6	49.59 ± 3.70	44.68 ± 4.62**	90.2 ± 6.6
WHR	0.88 ± 0.10	0.87 ± 0.08	100.1 ± 11.1	0.90 ± 0.04	0.88 ± 0.04	98.5 ± 5.9	0.93 ± 0.07	0.90 ± 0.07	97.7 ± 9.9
Glucose (mmol/l)	8.93 ± 2.11	7.34 ± 1.64*	84.4 ± 16.9	9.30 ± 2.65	7.07 ± 1.56**	77.4 ± 8.8	8.40 ± 2.57	7.01 ± 1.73	89.1 ± 30.1
Insulin (pmol/l)	26.43 ± 19.4	16.76 ± 10.9*	70.6 ± 24.7	26.20 ± 6.93	15.21 ± 5.92**	61.3 ± 26.1	31.48 ± 21.2	17.12 ± 12.0*	71.2 ± 73.8
Chol (mmol/l)	4.91 ± 1.03	3.72 ± 0.86**	75.9 ± 9.80†	4.84 ± 0.77	4.73 ± 0.82	97.8 ± 8.7	4.83 ± 0.77	4.54 ± 0.89	94.7 ± 14.9
HDL (mmol/l)	1.05 ± 0.21	0.81 ± 0.22**	73.1 ± 22.0††	1.12 ± 0.33	1.15 ± 0.31	104.0 ± 15.1	1.04 ± 0.24	1.08 ± 0.25	105.0 ± 17.3
LDL (mmol/l)	3.19 ± 1.03	2.23 ± 0.67**	71.4 ± 13.2††	2.82 ± 0.69	2.94 ± 0.77	107.2 ± 25.6	2.96 ± 0.65	2.90 ± 0.82	101.6 ± 33.4
TG (mmol/l)	1.43 ± 0.66	1.59 ± 0.69	117.6 ± 51.2	1.95 ± 1.39	1.38 ± 0.71	89.2 ± 33.0	1.79 ± 0.75	1.23 ± 0.50	74.0 ± 24.4
25(OH)D (nmol/l)	13.58±14.62	26.55±34.66	185.4±304.8	14.18±11.68	10.5±7	130.19±194.6	9.26±7.26	7.1±3.94	98.86±64.15

Data are means ± standard deviation. Within group statistical significance (pre to post-surgery) was determined using two-tailed paired t-test or Wilcoxon signed ranks test (*p<0.05; **p<0.01), whilst for between group comparisons one-way ANOVA or the Kruskal-Wallis test were used (†p<0.05; ††p<0.01). Change column (Δ%) denotes post-surgery values as percentage of pre-surgery values. EWL: excess weight loss, BMI: body mass index, WHR: waist-hip ratio, Chol: cholesterol, TG: triglycerides, HDL: High-density Lipoprotein, LDL: low-density lipoprotein, BPD: bilio-pancreatic diversion, LSG: laparoscopic Sleeve Gastrectomy, LAGB: laparoscopic adjustable gastric banding. All serum determinations correspond to fasting status.

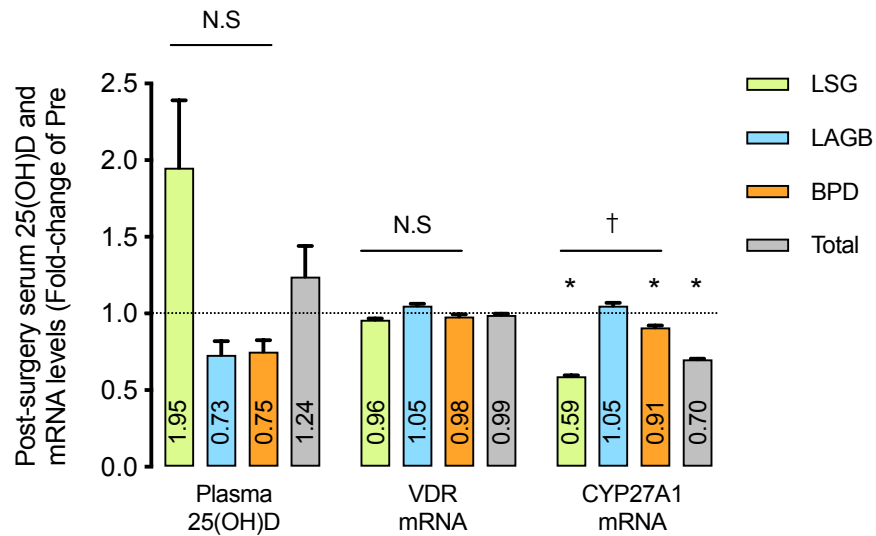


Figure 4.3.6 Effect of bariatric procedure and total weight loss on 25(OH)D and VitD metabolic components: Surgery-induced changes in circulating 25(OH)D and adipose tissue mRNA expression of VDR and CYP27A1 six months post-surgery. Bars represent means \pm standard error of the mean. Pre-to-post surgical differences were obtained via 2-tailed paired t-test ($*p < 0.05$). One-way non-parametric ANOVA (Kruskal-Wallis test) was used to determine differences between surgeries ($†p < 0.05$).

4.3.10 Relationship between body adiposity with VitD metabolic components

The association between changes in body adiposity post major weight loss with differences in VitD metabolic components and hence the improvements in circulating 25(OH)D levels was investigated. Spearman's analyses demonstrated significant correlations between body size and adiposity with the expression of CYP27A1. Percentage body fat and total body weight were directly associated with gene expression of CYP27A1 (Figure 4.3.7 (A-B)). Moreover, plasma 25(OH)D was identified to have a significant association across all surgeries with CYP27A1 gene expression. This relationship show that lower expression of CYP271A was associated with higher total circulating 25(OH)D (Figure 4.3.7 (C)). However, no significant associations were observed between the expression of VDR and circulating 25(OH)D or any of the anthropometric measures.

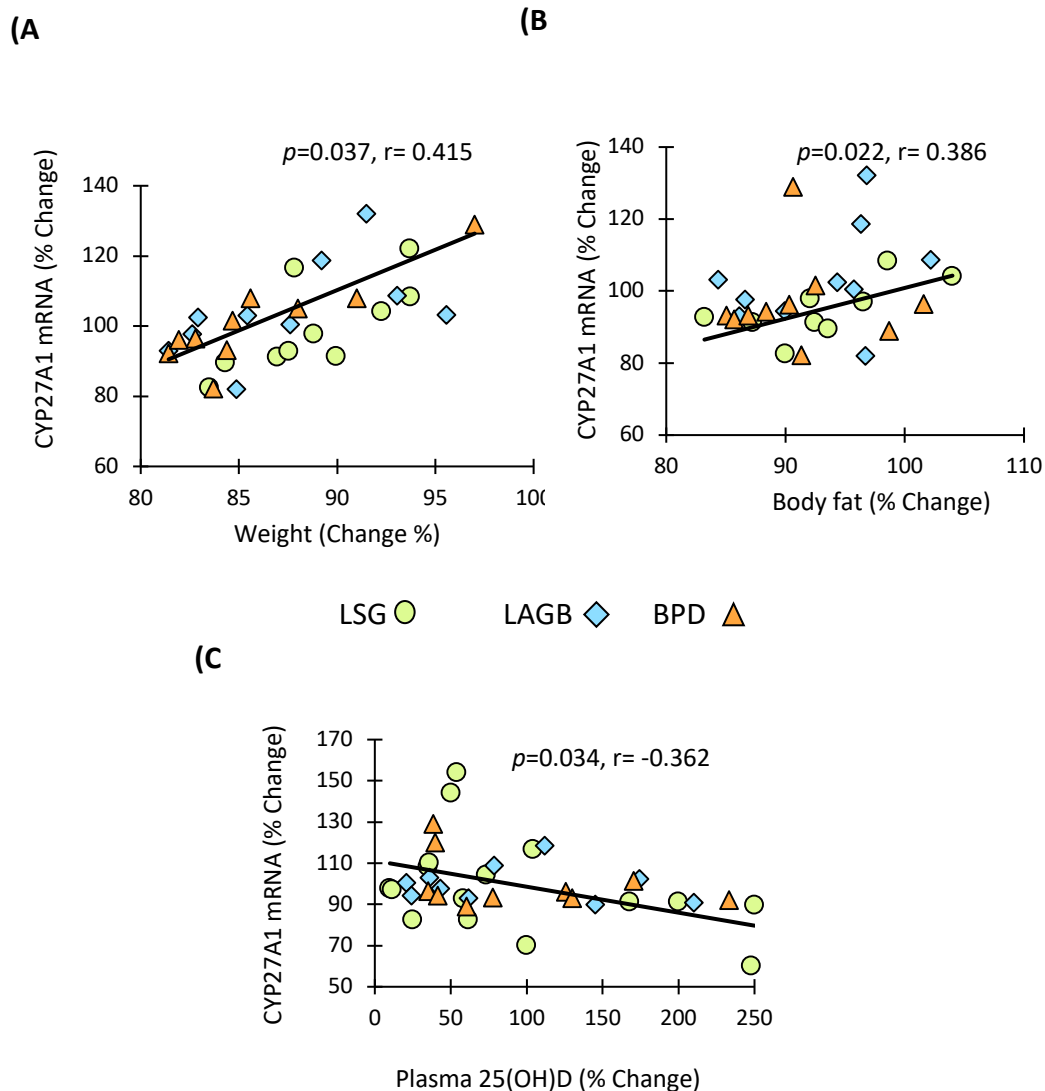


Figure 4.3.7 Association of AT CYP27A1 gene with body adiposity and plasma 25(OH)D: Scatter plots of CYP27A1 gene correlated against A) body weight, B) percentage body fat, and C) total circulating 25(OH)D. Correlations were calculated using change variables (percentage of pre levels) in the entire patient cohort (n=35), but individual data points are color-coded according to surgical procedure: Bilio-pancreatic diversion (BPD), laparoscopic sleeve gastrectomy (LSG) or laparoscopic adjustable gastric banding (LAGB). Linear trend line is also shown with Pearson correlation statistic (r) and significance (p).

4.4 Discussion

The main aim of this study was to investigate whether low circulating VitD levels observed in obesity are due to altered VitD metabolism in AT, and whether major weight loss could lead to improvements in VitD metabolism within AT and hence improvements in VitD circulating levels. These investigations revealed four key findings. Firstly, expression of VDR and all VitD metabolizing enzymes were raised in overweight and obesity in a depot specific manner for each gene. Secondly, AT contents of VitD metabolic components were associated with circulating levels of VitD. Thirdly, all VitD metabolic components were positively associated with inflammatory mediators which were associated with circulating 25(OH)D. Finally, major weight loss resulted in decreased expression of VitD hydroxylase in AT which was associated with improvements in circulating 25(OH)D levels.

In humans, CYP2R1 is known to be the key enzyme that converts VitD to its circulating form 25(OH)D, then 25(OH)D binds to VDR which initiates VitD actions [463]. These current findings showed that the expression of VDR and CYP2R1 were significantly higher in AbdSc fat than Om AT and along with AbdSc CYP27A1, these were inversely correlated with circulating 25(OH)D levels ($p = 0.006$ $r = -0.279$, $p = 0.047$ $r = -0.209$ and $p = 0.001$ $r = -0.289$ respectively). These findings suggest that AbdSc AT might be more active source for VitD metabolism and hence circulating levels are more affected by AbdSc contents of VitD metabolic components than Om tissue contents. The present findings appear to be consistent with a very recent study by Lauriane et al. [221] who observed that

25(OH)D content in AT was inversely correlated to the circulating levels of 25(OH)D and that this accumulation of 25(OH)D in AT was highly correlated to the induction of CYP2R1 in AbdSc AT; which could actively participate in VitD3 trapping and subsequent conversion to 25(OH)D in AT. These data might suggest the metabolism of VitD within AT and that these increases in VDR and CYPs are due to more VitD sequestration in AT as supported by several studies [221, 267, 279, 411, 412, 468-470]. In contrast, in Om AT there was higher expression of CYP2J2 among all weight groups and higher expression of CYP27A1 in lean participants. Only Om CYP27A1 showed a significant linear association with circulating 25(OH)D levels. This association aligns with the finding of Wamberg et al. [220] who noted a positive correlation between visceral CYP27A1 with circulating 25(OH)D. The negative association between VDR in AbdSc depot and circulating 25(OH)D might be attributed to compensatory mechanism for circulating VitD deficiency as suggested by Daniel *et al* [461]. Yet the subcutaneous fat contribution to the absolute changes in circulating VitD levels remains to be clarified.

Low circulating 25(OH)D levels were apparent in obese and overweight patients and were associated with higher BMI among the total population which is in line with data reported by numerous studies [246, 427, 471, 472]. The data in this study showed that obese and overweight individuals (who had lower circulating levels) had significantly higher expression of VDR and the two 25-hydroxylases CYP2J2 and CYP27A1 compared to lean. These findings however, were only observed in AbdSc fat and no specific trend was noted in Om AT. This

could point toward the importance of AbdSc for VitD levels as it accounts for about 85% of body fat although Om AT is considered to be more metabolically active [473, 474]. Contrary to our findings, VDR and CYP2J2 have been observed to be elevated in lean compared with obese subjects in a cross-sectional study and this discrepancy could be due to differences between study populations [220]. Further analysis of VitD metabolism was investigated in a study where male mice were treated with a high fat diet for 11 weeks, which led to a body fat increase. There was a significant increase in VDR expression in AT, which suggested a local activation of VitD signalling during the obese state [221] and confirms that VDR is a key promoter of adipogenesis [263]. VitD hydroxylase CYP27A1 has also been found to be elevated in mature adipocytes compared with pre-adipocytes [475] which may suggest the existence of VitD hydroxylation in adipose tissue [305, 476].

The findings of this study confirmed that VDR gene expression in both fat depots is strongly associated with all inflammatory cytokines including CD14, CD68, IL-1 β , IL-6, IL-8, MCP1, NF κ β , TNF-alpha. As shown in findings of this and other studies, TNF-alpha can activate the expression of VDR gene and low VitD levels can lead to the activation of inflammatory pathways, which in turn promotes the expression of VDR [147]. Raised expression of VDR in the absence of its ligand has been linked with opposite effects in gene expression regulation [477, 478]. An experimental study has created a form of VDR gene that is unable to bind with its ligand VitD and have activated VitD by a synthetic ligand. Results showed that VDR was capable of suppressing the expression of Trpv6 and

Slc30a10 genes responsible for calcium and manganese absorption from intestine [477, 478]. However, VitD administration has been reported to downregulate VDR gene expression [460] which has been confirmed with the negative correlation we found between these two parameters.

Although there is a lack of data on the link between VitD hydroxylases and inflammation, Stoffels K. *et al*, [311] showed that stimulation of the TLR4/CD14 receptor complexes by lipopolysaccharide induces the expression of the enzyme responsible of VitD activation (1-alpha-hydroxylase). Moreover, it is been shown that the local synthesis of these enzymes is increased by inflammatory cytokines leading to an increase of the local synthesis of VitD to modulate or inhibit inflammatory status [305, 465]. In vitro studies have also showed that the expression of CYP2R1, CYP2J2 and CYP27A1 is affected by inflammatory cytokines whereas the expression of CYP27B1, for example, is strongly controlled by PTH and 1,25(OH)₂D₃ in the kidney [479]. According to the present data, all VitD metabolic components were found to be positively associated with most inflammatory cytokines which might point toward a local synthesis of 25(OH)D [279, 469] which was positively associated with BMI but negatively with plasma 25(OH)D.

The inverse associations observed between plasma 25(OH)D and inflammatory mediators within AT are affirmed by numerous studies [480-484]. Several mechanism for these anti-inflammatory effects were suggested, including inhibition of the NFκβ [299, 381] and mitogen activated protein kinase signalling pathways [147]. The increases in inflammatory mediators and VitD metabolic

components during obesity that are associated with lower plasma levels might explain the need of VitD monitoring especially for this group.

Further analysis explored the role of weight loss over time with changes in circulating VitD and its metabolic components in AbdSc AT following bariatric surgeries. Circulating 25(OH)D increased post weight loss (although none of participants were on VitD supplementation), and changes in circulating levels were associated with changes in CYP27A1 gene expression in AT. Post-surgery weight loss resulted in significant decrease in the expression of CYP27A1 and changes in this enzyme were significantly associated with changes in overall weight and fat mass. These data might indicate reduced VitD metabolism within AT which could be a result of less sequestration in AT following reduced weight and fat mass and that increased VitD hydroxylases in AT during obesity may indicate a direct trapping of 25(OH)D explaining the reduced circulating 25(OH)D [221, 411, 412]. The present findings are in accord with a previous study that showed a significant increase in VitD metabolic components within AT after 11 weeks of high fat diet and these increases were consistent with AT VitD content and negatively associated with circulating VitD [221]. It is also important to note that six months post-operatively subjects were still obese, which may explain why expression of the VDR did not change and that subjects might need to lose more of their excess weight and be classified as overweight or lean to show differences in VDR gene expression.

The strength of current studies is the collection of a large sample size with all weight groups (lean, overweight and obese) of paired SC and OM fat depots.

Furthermore, the collection of samples of AbdSc both pre and post significant weight loss allowed exploration of longitudinal changes in VitD status and metabolism. Unfortunately, samples of Om AT were not available from the weight loss trial, which could have provided additional information. A drawback of these studies is that we did not have the data on how much sunshine the participants were exposed to or the seasons when the samples were collected.

In conclusion, based on the present findings, it could be considered that AT is not only a storage pool for VitD, but it is also involved in VitD metabolism (the hydroxylation of cholecalciferol by 25-hydroxylases into the circulating form 25(OH)D). These studies have highlighted that obese women had significantly higher expression of VitD hydroxylases in AT, yet associated with lower circulating 25(OH)D levels. Furthermore that in participants with raised circulating VitD levels there was an associated decrease of VitD hydroxylase (CYP27A1) expression in AT; with CYP27A1 being linked to changes in overall weight and body fat mass post major weight loss. Accordingly, the data suggests that disturbed VitD metabolism in obese people could be a reversible state and that individuals with substantial weight gain, particularly increased fat mass, may require higher doses of VitD to reach acceptable circulating levels to potentially moderate AT VitD hydroxylase levels to improve health outcomes. Finally, the demonstrated *in vivo* correlations between 25(OH)D and different inflammatory mediators informed the question what direct influence may VitD have on adipocyte metabolism to improve metabolic health, this will be explored in the following chapter.

Chapter 5

VitD reduces the inflammatory response and restores glucose uptake in insulin resistant adipocytes

5.1 Introduction

As outlined in Chapter 1, obesity is an increasing common global public health problem [396, 485], characterized by an expansion of white adipose tissue (AT) [100] which is associated with increased infiltration of macrophages leading to low-grade inflammation and insulin resistance [294]. Histologically, AT is considered as an endocrine organ, which is capable of secreting a number of protein factors that are directly involved in inflammation [486-488]. During obesity, the expression and/or release of insulin and some inflammatory factors, including TNF-alpha, IL-1 β , IL-6, monocyte chemoattractant protein-1 (MCP-1) and IL-8, have been shown to be elevated [489-491] which might explain the relationship between obesity and multiple metabolic diseases including T2DM [146, 492, 493]. Production of local inflammatory factors does not only impact adipocyte cellular insulin sensitivity [494-496] but also influences systemic glucose metabolism via targeting insulin signalling in insulin-sensitive organs, in other tissues such as muscles and the liver [146]. In parallel to the developing obesity epidemic, low VitD levels have also been identified as a major public health issue particularly among people with obesity, as it is estimated that more than 84% of obese subjects have low levels of VitD [247]. For a long time, VitD was believed to have a role confined to the metabolism of calcium and phosphorus [497, 498]. Now, VitD is considered to be a multifunctional factor whose circulating concentration appears to be linked to the prevalence of several diseases including obesity [237] and T2DM [191] and higher levels been associated with better health outcomes during obesity as shown in several studies [50, 256, 262, 368, 415, 448, 471].

Molecular studies have highlighted the link between adipose tissue inflammation and insulin resistance through the up-regulation of two pathways linking the two: nuclear transcription factors NF-kappa B (NFκβ) [499, 500] and mitogen-activated protein kinases (MAPKs) pathways which involve p38 and extracellular signal regulated kinases (ERK1/2) [334, 501]. However, the activation of MAPKs is also able to activate NFκβ which is a master regulator of inflammation and insulin resistance [333]. For example, in primary mouse adipocytes, high fat diet resulted in increased expression of NFκβ which was dependent on the ERK pathway [333]. Moreover, MAPKs mediated the production of pro-inflammatory cytokines including IL-1β, TNF-alpha and IL-8 following hyperosmolar stress in epithelial cells [501]. The activity of ERK1/2 has been found to be increased in human and mouse AT in the obese and diabetic state [502-504]. Furthermore, inhibition of p38 and ERK signalling during insulin resistance have completely restored insulin-stimulated glucose uptake and insulin signalling in 3T3 adipocytes and muscle cells [333, 334]. Hence, targeting MAPKs pathway has been suggested as a potential treatment option to reduce insulin resistance through promoting AKT phosphorylation and GLUT4 translocation from an intracellular compartment to the plasma membrane [333, 334]. Therefore, it was important to investigate the effect of VitD on MAPKs signalling which mainly include ERK1/2 and p38 MAPK.

The beneficial effects of VitD on different metabolic diseases are believed to be the result of the VitD regulatory effects on several metabolic pathways which regulate inflammation and insulin signalling [290, 381, 382]. This appears

to occur as VitD can prevent the production of pro-inflammatory cytokines via targeting MAPKs and NFκβ signalling pathways in human adipocytes and peripheral blood mononuclear cells (PBMC) [290, 381]. From the previous findings detailed in Chapter 3, baseline VitD levels are associated with T2DM remission post bariatric surgery and explained 10% of divergence in amounts of weight regained post-bariatric surgery. This is supported by *in vitro* studies which showed the involvement of VitD in adipogenesis which may explain the link between VitD and obesity [263]. Thus, it could be proposed that VitD positively impacts AT physiology and prevents some metabolic disorders including insulin resistance and T2DM. However, the preventive effects of 1,25(OH)₂D₃ from insulin resistance and inflammation through its involvement in different pathways has not previously shown.

Based on current evidence the present study sought to investigate the impact of VitD and, more particularly, the active form (1,25(OH)₂D₃) on inflammation and glucose uptake in adipocytes under conditions of inflammation and insulin resistance using two biological models. These studies explored the hypothesis that the observed negative health associations of low VitD levels are mediated via an impaired inhibition of cytokine transcription and insulin signalling leading to metabolic disturbances. Therefore, in this study, the preventive effects of 1,25(OH)₂D₃ on expression of inflammatory cytokines and insulin resistance was evaluated.

5.2 Methods

5.2.1 Cell culture

SGBS cells [377] were cultured and differentiated as detailed in chapter 2 – section 2.3.2. Oil Red-O staining was carried out as described in chapter 2 – section 2.5.1 at days 0,5,10 and 14 of the differentiation process to ensure that the pre-adipocyte SGBS cells had differentiated into mature adipocytes. Two established *in vitro* models of insulin resistance were used in this study; chronic TNF-alpha (5ng/ml) for 24 hr and chronic high glucose (HG) (25mM) plus high insulin (HI) (100nM) (HG/HI) for 24 hr [299, 383, 385, 387, 388]. All treatments were initiated on day 14 of differentiation where cells were ~80% differentiated. Differentiated SGBS cells were washed with PBS and changed to serum-free, low-glucose (1 g/l) DMEM supplemented with 0.01 mg/ml transferrin for 24 hr. To investigate the effect of VitD on inflammation and insulin signalling, mature adipocytes were treated with 1,25(OH)₂D₃ (10nM and 100nM) for 24 hr and the control received no treatment. Cells were incubated with either chronic TNF-alpha (5ng/ml) for 24 hr or chronic HG (25mM) plus HI (100nM) (HG/HI) for 24 hr. To examine the preventive effects of VitD from inflammation and IR, cells were preincubated with 1,25(OH)₂D₃ (10nM and 100nM) for 24 hr then treated with either TNF-alpha or HG/HI in the presence of 1,25(OH)₂D₃ (10nM and 100nM) for another 24 hr.

Following treatments, cells were harvested and RNA and protein extraction were performed to allow analysis by Western blot and RT-PCR. Six samples for each treatment were collected, processed and analysed in order to

increase the reliability of the results. Glucose uptake assay was performed as detailed in chapter 2 – section 2.5.4.

5.2.2 Immunofluorescence (IF)

SGBS cell were seeded onto 10 g/ml fibronectin (Invitrogen)-coated glass coverslips kept in 24 wells. After reaching confluence, the cells were differentiated into adipocytes and treated with previously described treatments. IF was performed with prewashing and post-washing with PBS for each step, fixing of cells for 30 min at room temperature in 4% formaldehyde, and then permeabilization for 5 min with 0.1% Triton X-100 in PBS for NF κ B assay. For GLUT4 IF the cells were not permeabilised. Nonspecific binding sites were blocked with blocking solution (Goat serum (fc 5%) and 10% Saponin (fc 0.1%) in PBS) for 1 hr at RT. Cells were incubated with rabbit anti-NF-kB p65 or anti-GLUT4 primary antibodies overnight and Alexa Fluor 488 goat anti-rabbit IgG for 1 hr in blocking solution. After incubation with DAPI at 1 mg/ml for 5 min, coverslips mounted using Immunomount medium. Fluorescence images were acquired by confocal microscope at 40x magnification through an oil objective lens.

5.2.3 sCD14 determination

sCD14 levels were determined using the Quantikine ELISA human CD14 Immunoassay (R & D Systems) following the manufacturer's protocol. Sensitivity for CD14 was 125 pg/ml and the intra-assay coefficient of variation was 5.5% while the inter-assay coefficient of variation was 6.3%.

5.2.4 Statistical analysis

The data are the mean of three or two independent experiments each performed in six samples. The data are reported as the mean \pm standard error of the mean (SEM). Significant differences between the control and treated group were determined using ANOVA followed by the Tukey-Kramer post hoc test or the unpaired Student's t-test. Statistical analyses were performed using the SPSS 25.0 software. *p* value was considered significant if <0.05 .

5.3 Results

5.3.1 Inhibitory effect of 1,25(OH)₂D₃ on TNF-alpha and HG/HI stimulated expression of inflammatory mediators in human adipocytes

SGBS cells were differentiated into adipocytes as described in chapter 2. In this cellular model, ~80% of the cells were differentiated and lipid droplet accumulation was confirmed by Oil Red O staining (Figure 5.3.1). In these experiments, the impact of 1,25-(OH)₂D₃ on the expression of markers of inflammation and metabolic health was evaluated. Expression of MCP-1, IL-1 β , IL-6, IL-8 and CD14 were used as representative markers for inflammation and metabolic syndrome.

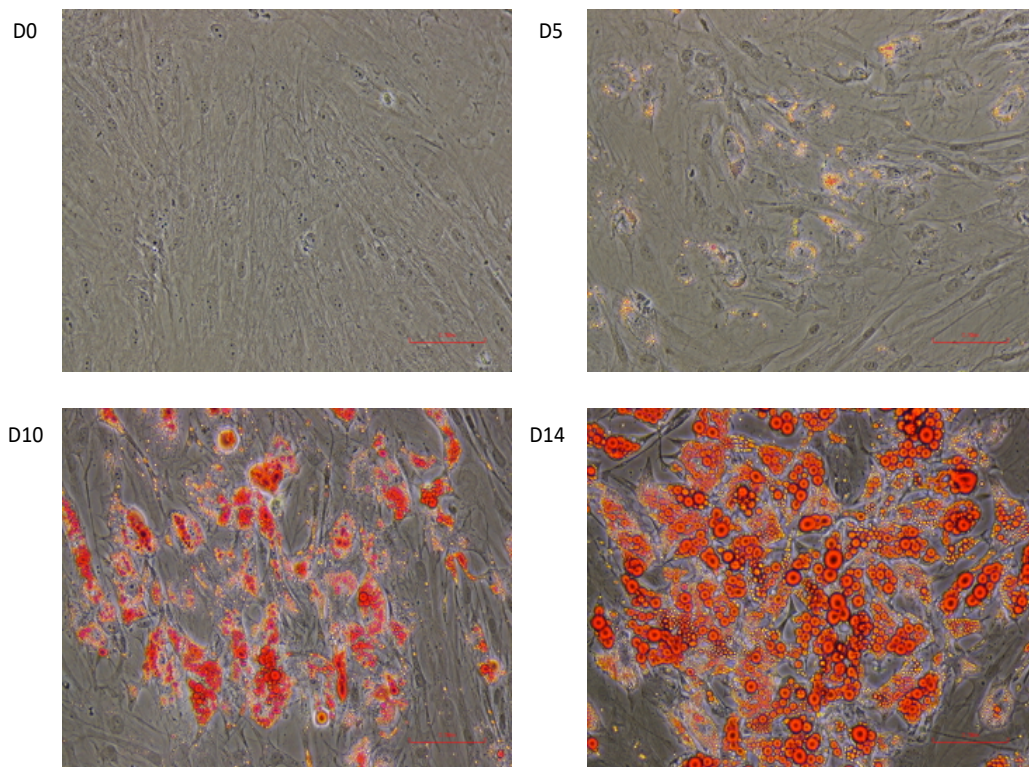


Figure 5.3.1 Adipocyte Staining: Oil Red-O staining of SGBS cells at Day 0, 5, 10 and 14 of differentiation show a build-up of stained lipid droplets at the end of the differentiation process, indicating cells have developed into mature adipocytes X20 view, scale bar represents 200 μ m.

As the actions of $1,25(\text{OH})_2\text{D}_3$ are mediated through its receptor VDR which modulates the transcription of a number of target genes including the ones involved in inflammation and IR, a confirmation step for the presence of VDR gene in SGBS mature adipocytes was investigated. Expression of VDR mRNA in SGBS mature adipocytes was compared to the expression of VDR mRNA of lean and obese samples of human AbdSc AT and primary adipocytes. One-way ANOVA showed no overall significant differences of VDR mRNA within the groups ($p=0.213$) (Figure 5.3.2).

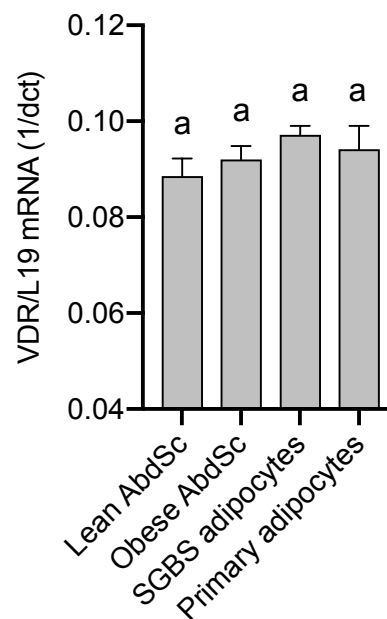


Figure 5.3.2 Expression of VDR mRNA in adipocytes: VDR mRNA was quantified by qRT-PCR and compared between AbdSc AT of 11 lean, 11 obese, 6 samples of SGBS mature adipocytes (day 14) and 6 samples of mature primary adipocytes (day 14). Bars represent means \pm standard error of the mean. One-way ANOVA (using Tukey-Kramer post hoc test) showed no overall significant differences of VDR mRNA within the groups ($p > 0.05$)

In order to ascertain whether VitD can prevent metabolic syndrome during obesity, mature adipocytes were preincubated with 10nM or 100nM of the active form of VitD (1,25-(OH)₂D₃) for 24 hr prior to treatments with TNF-alpha (5ng/ml) or HG (25mM) plus HI (100nM) (HG/HI) in the presence of 1,25-(OH)₂D₃ for another 24 hr. Results showed that expression levels of MCP-1, IL-1 β , IL-6 and IL-8 were significantly increased with TNF-alpha (5ng/ml) only treatment by 423, 4.7, 4.5 and 2364-fold, respectively (Figure 5.3.3). Pre-incubation with 1,25-(OH)₂D₃ resulted in significant reductions in MCP-1 (19.3% with 10nM and 29% with 100nM), IL-1 β (54% with 10nM and 54.7% with 100nM which restored it to control levels) and IL-8 (40% with 10nM and 61.5% with 100nM) expression levels (Figure 5.3.3). The second model was the insulin resistance condition model (HG/HI). Findings highlighted that IL-1 β was significantly increased by 23.2-fold ($p < 0.001$) and this was decreased by 55.7% when preincubated with 1,25-(OH)₂D₃ for both concentrations ($p < 0.001$). However, no significant changes were observed in MCP-1, IL-6 and IL-8 expression levels (Figure 5.3.3). TNF-alpha treatment of adipocytes resulted in 3.3 fold increase in expression levels of VDR mRNA and these levels were attenuated by 37.5% ($p < 0.001$) and 42.5% ($p < 0.001$) when preincubated with 10nM and 100nM of 1,25-(OH)₂D₃ respectively. However, no significant changes were observed in VDR mRNA expression levels within HG/HI model (Figure 5.3.3). No significant changes in the expression levels of inflammatory genes were observed with 1,25-(OH)₂D₃ treatment only of either concentrations (Figure 5.3.3).

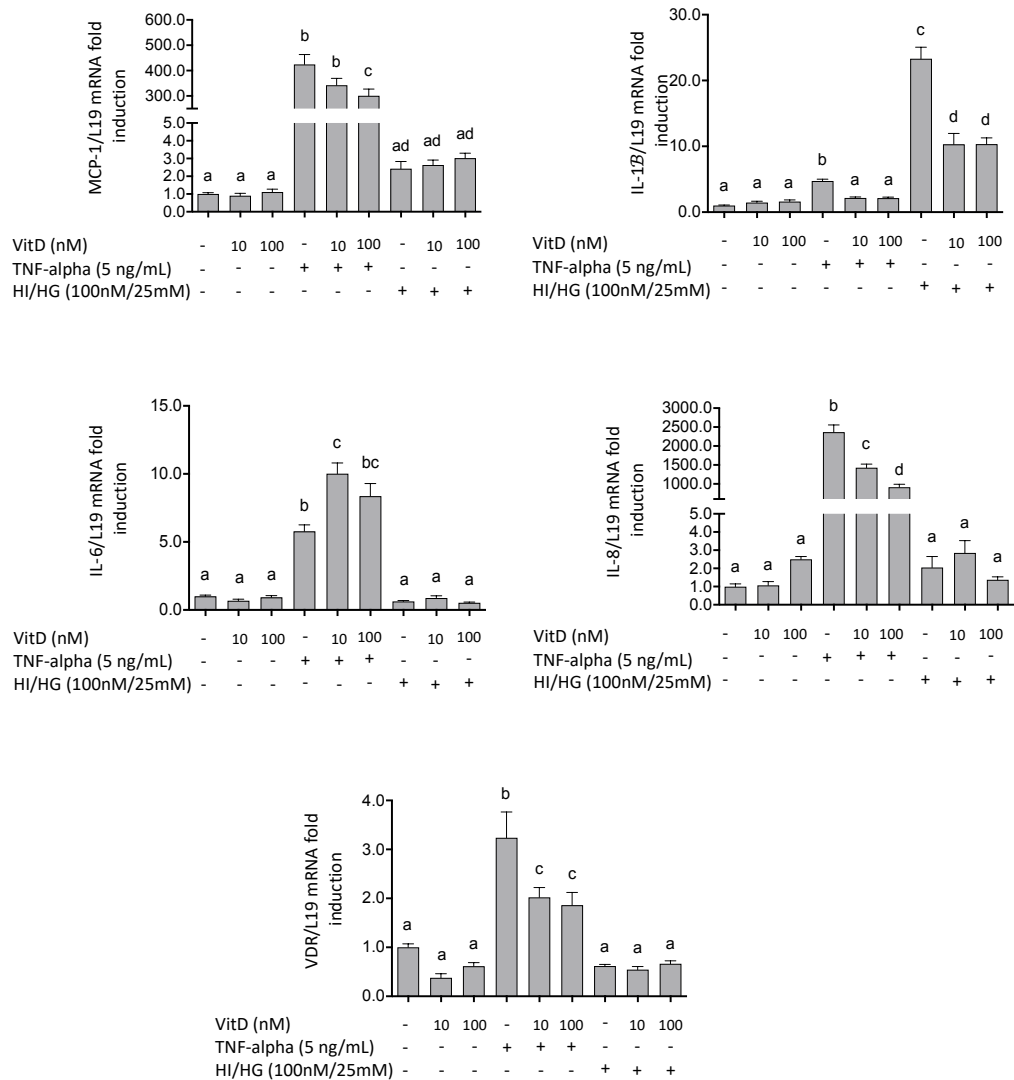


Figure 5.3.3 Effect of 1,25-(OH)₂D₃ on markers of inflammation: Mature SGBS human adipocytes were preincubated with 1,25(OH)₂D₃ (10nM or 100 nM) for 24 hr, followed by TNF-alpha (5ng/ml) or high glucose (25mM) plus high insulin (100nM) (HG/HI) stimulation for another 24 h. mRNA Expression levels of MCP-1, IL-1β, IL-6, IL-8 and VDR were quantified by qRT-PCR. The data are expressed as a relative expression ratio to the controls. Bars represent means ± standard error of the mean of three independent experiments done in six samples for each treatment. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test *p* < 0.05.

A 20 and 56 fold increase in CD14 expression levels was reported with combined treatment of TNF-alpha plus 10nM and 100nM of 1,25-(OH)₂D₃

respectively ($p < 0.001$). In the HG/Hi model, the preincubation with 10nM and 100nM of 1,25-(OH)₂D₃ resulted in 34 and 71 folds increase in CD14 expression levels respectively ($p < 0.001$) (Figure 5.3.4 (A)). Interestingly, no changes in CD14 expression levels been observed in both models without 1,25-(OH)₂D₃ pre-incubation (TNF-alpha or HG/Hi only treatments) or with 1,25-(OH)₂D₃ only treatment (Figure 5.3.4 (A)). Therefore, the innate immune-related receptor TLR4 was measured in these cells (since CD14 is a co-receptor for TLR4). It was found that SGBS preadipocytes and adipocytes expressed significantly less TLR4 compared with all cell types tested ($p < 0.05$) including: primary preadipocytes, mature adipocytes, and lean and obese omental and subcutaneous adipose tissue (Figure 5.3.5). Furthermore, in order to confirm the initiated anti-inflammatory pathway, the release of soluble CD14 was measured in the condition media. Data from ELISA assay showed that the release of sCD14 was increased by 42% ($p < 0.005$) and 71% ($p < 0.001$) when TNF-alpha and HG/Hi were combined with 1,25(OH)₂D₃ respectively. No changes were observed in sCD14 levels in without 1,25-(OH)₂D₃ pre-incubation (TNF-alpha or HG/Hi only treatments) or with 1,25-(OH)₂D₃ only treatment (Figure 5.3.4 (B)).

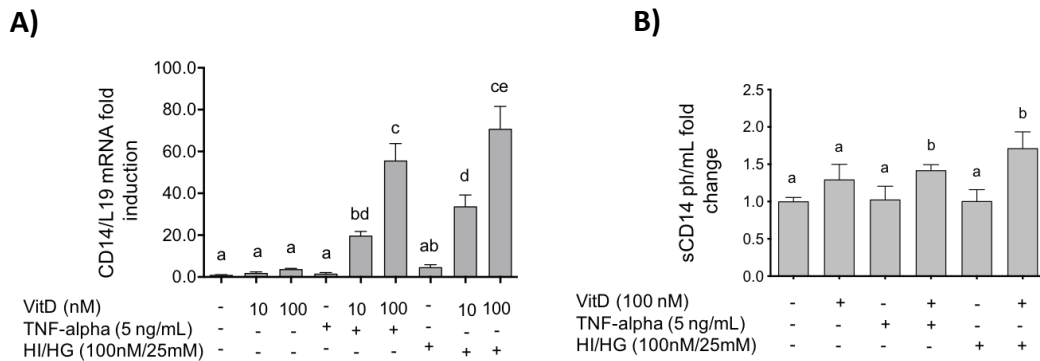


Figure 5.3.4 Figure 3.4 Effect of 1,25-(OH)2D3 on CD14 mRNA and secretion: Mature SGBS human adipocytes were preincubated with 1,25(OH)2D3 (10nM or 100 nM) for 24 h, followed by TNF-alpha (5ng/ml) or high glucose (25mM) plus high insulin (100nM) (HG/HI) stimulation for another 24 h. A) mRNA Expression levels of CD14 were quantified by qRT-PCR. B) The concentration of soluble CD14 was measured in condition media by a sandwich enzyme-linked immunosorbent assay (ELIZA). The data are expressed as a relative expression ratio to the controls. Bars represent means \pm standard error of the mean of three independent experiments done in six samples for each treatment. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$.

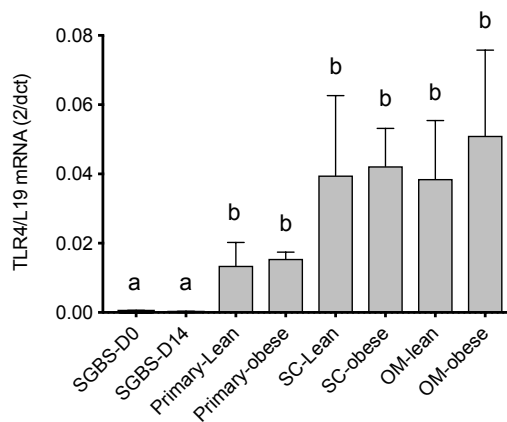
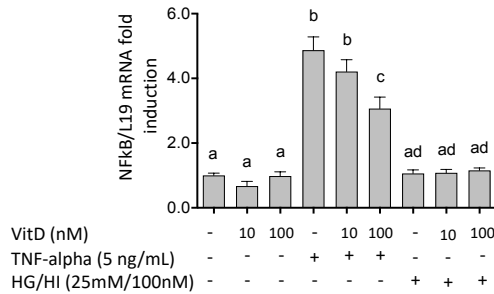


Figure 5.3.5 expression of TLR4 in SGBS cells and different pre/mature adipocytes: TLR4 gene was measured in SGBS preadipocytes and mature adipocytes then was compared to the expression levels of TLR4 in different adipocytes, primary preadipocytes, mature adipocytes, and lean and obese omental and subcutaneous adipose tissue. Expression levels of TLR4 mRNA were quantified by qRT-PCR. Bars represent means \pm standard error of the mean of three samples. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$.

5.3.2 Inhibition of TNF-alpha and HG/HI-induced inflammation gene expression by 1,25(OH)₂D₃ is mediated by inhibition of NFκβ

There is a large amount of evidence that NFκβ activation correlates with inflammation [381, 499, 500, 505-507]. In the current study, it was hypothesized that in SGBS human adipocytes, the anti-inflammatory effect of VitD on inflammation induced by TNF-alpha or HG/HI condition is mediated by an effect of VitD on NFκβ. Data revealed that SGBS cells expressed higher NFκβ mRNA by 4.5 fold ($p < 0.001$) with TNF-alpha stimulation and this was reduced 13% and 37% ($p < 0.005$) when preincubated with 1,25(OH)₂D₃ of both 10nM and 100nM, respectively (Figure 5.3.6 (A)). No changes were observed in HG/HI model in all treatments (Figure 5.3.6 (A)). The mRNA expression responses were paralleled by TNF-alpha-stimulated NFκβ induction of NFκβ protein expression levels. NFκβ protein expression levels were elevated by 1.35-fold ($p < 0.05$) when stimulated with TNF-alpha for 24 hr, while pre-treatment and combination with 1,25(OH)₂D₃ (100nM) resulted in 28% decline in protein levels ($p < 0.05$) (Figure 5.3.6 (B)). Interestingly, protein expression levels of NFκβ were significantly decreased by 51% with HG/HI treatment and by 39% when preincubated with 1,25(OH)₂D₃. No changes been observed between HG/HI treatment only and HG/HI treatment with 1,25(OH)₂D₃ preincubation (Figure 5.3.6 (B)). IF also showed that NFκβ p65 protein expression was remarkably upregulated by TNF-alpha treatment, and that preincubation and combination with 100nM 1,25(OH)₂D₃ inhibited this effect. However, no changes were observed in NFκβ p65 protein expression with HG/HI model (Figure 5.3.7).

A)



B)

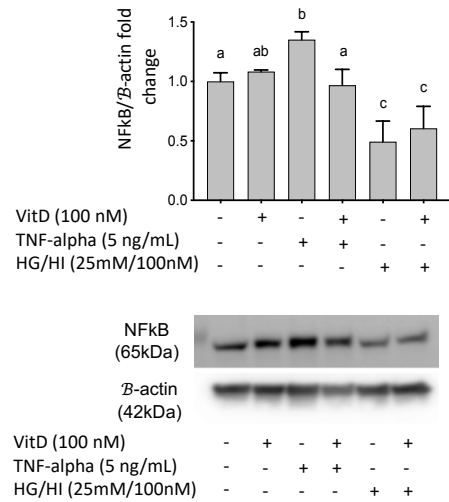


Figure 5.3.6 Effect of 1,25(OH)2D3 on NF-κB expression in mature SGBS adipocytes: Mature adipocytes were preincubated with 1,25(OH)2D3 (10nM or 100 nM) for 24 h, followed by chronic TNF-alpha (5 ng/ml) or high glucose (25mM) plus high insulin (100nM) (HG/HI) stimulation for another 24 h. (A) expression levels of NF-κB mRNA were quantified by qRT-PCR. (B) Representative WB of SGBS mature adipocytes for protein expression and β-actin was used as the endogenous control. Expression values in control were set at one to assess fold change from this value in other treatments. Bars represent means ± standard error of the mean of three independent experiments done in six samples for each treatment. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$.

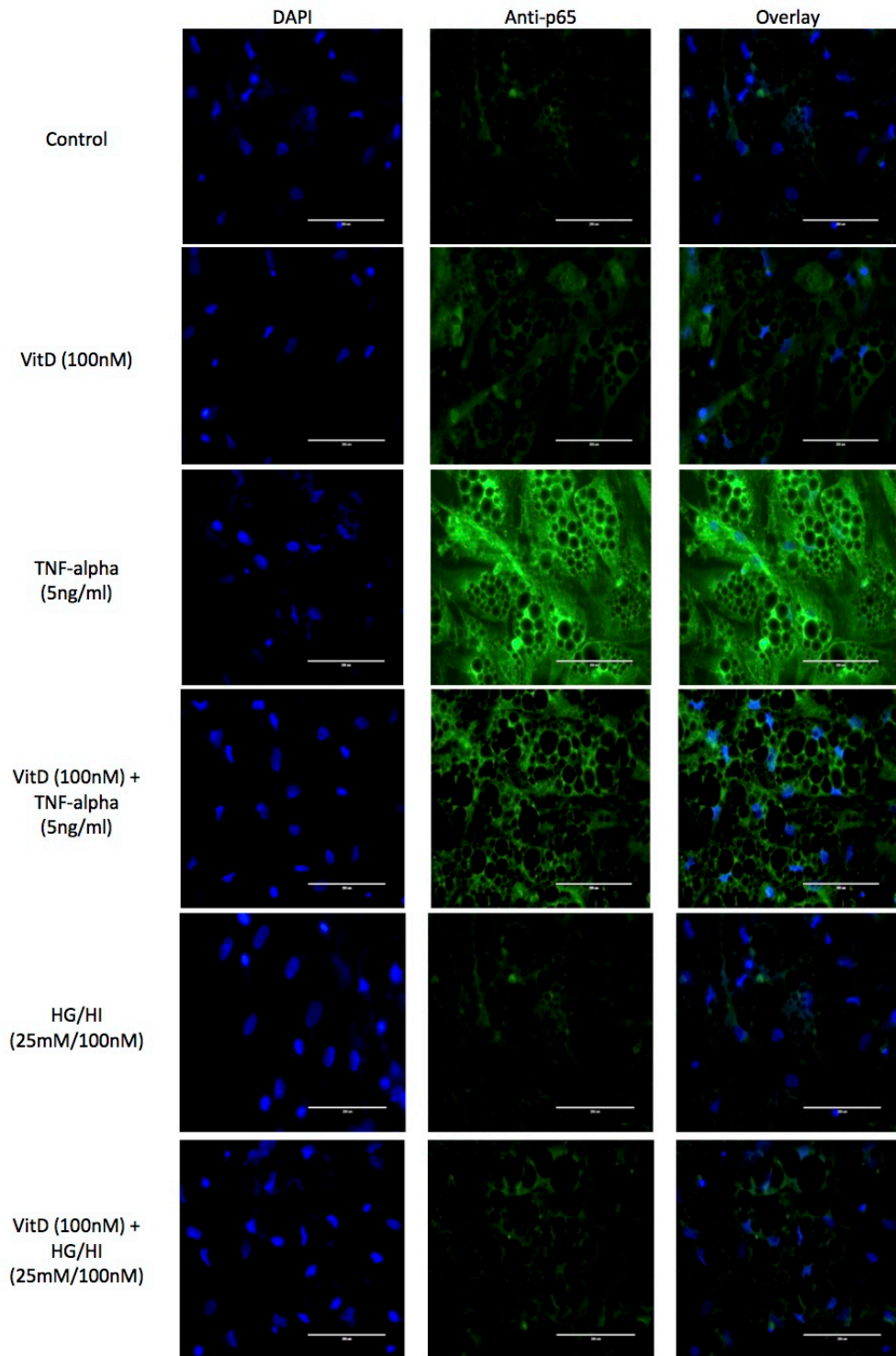
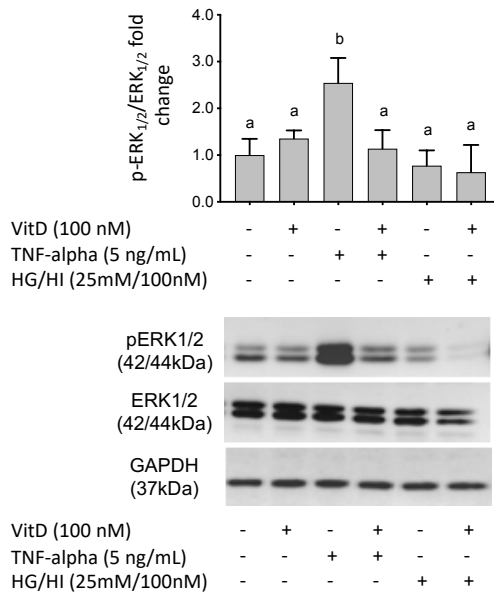


Figure 5.3.7 Fluorescence Microscopy of Mature SGBS Adipocytes preincubated with 1,25(OH)₂D₃, followed by TNF-alpha or high insulin treatments: Mature SGBS adipocytes were preincubated with 1,25(OH)₂D₃ (100 nM) for 24h, followed by TNF-alpha (5ng/ml) or high glucose (25mM) plus high insulin (5ng/ml) (HG/HI) for another 24h. Expression of the NFκβ subunit p65 was determined by IF detection using primary and secondary antibodies, anti-NFκβ p65 and goat anti-rabbit IgG (Alexa Fluor 488), respectively. Nuclei were stained with DAPI, and photomicrographs were obtained using a fluorescence microscope at 40x magnification, scale bar represents 200 μm.

5.3.3 Stimulated MAPK signalling is modulated by 1,25(OH)₂D₃ via inhibition of ERK1/2 and P38 phosphorylation

This study investigated whether the mitogen-activated protein kinase (MAPK) pathway is activated by TNF- α and HG/HI treatments in SGBS cells and whether 1,25(OH)₂D₃ may be involved in preventing their effect by inhibiting MAPKs' extracellular signal regulated kinase (ERK) and p38. P-ERK1/2 and p-p38 were activated (phosphorylated) in SGBS adipocytes after 24 hr of TNF- α (5ng/ml) treatment. When normalized by total ERK1/2, the quantified ratio of p-ERK_{1/2}/ERK_{1/2} indicated that TNF- α resulted in 2.5-fold increase ($p < 0.005$) in p-ERK1/2 and this effect was restored to control levels ($p < 0.001$) when preincubated with 1,25(OH)₂D₃ (Figure 5.3.8 (A)). Levels of p-p38 were also stimulated by 1.43-fold ($p < 0.05$) with TNF- α treatment and this was inhibited by 37% ($p < 0.05$) with 1,25(OH)₂D₃ preincubation (Figure 5.3.8 (B)). However, no significant changes been observed in levels of p-ERK1/2 and p-p38 in HG/HI model (Figure 5.3.8 (A and B)).

A)



B)

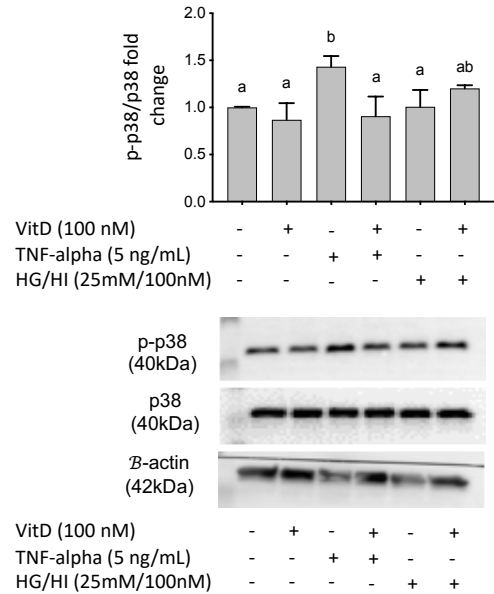


Figure 5.3.8 Preventive effect of 1,25(OH)₂D₃ on MAPK pathway activation: Representative western blots from two experiments performed in six samples for each treatment showing (A) the bands of p-ERK1/2, total ERK1/2, and quantified ratios (mean ± standard error of the mean) of the bands densities of p-ERK1/2 by dividing band densities of p-p38 over the total and β-actin was used as the endogenous control, (B) the bands of p-p38, total p38, and quantified ratios (mean ± standard error of the mean) of the bands densities of p-p38 in SGBS mature adipocytes preincubated with 1,25(OH)₂D₃ (100 nM) for 24h, followed by chronic TNF-alpha (5ng/ml) or chronic high glucose (25mM) plus high insulin (5ng/ml) (HG/Hi) for another 24h. Expression values in control were set at one to assess fold change from this value in other treatments. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$.

5.3.4 VitD improves insulin sensitivity by stimulating AKT phosphorylation and GLUT4 expression/translocation

The impact of the two insulin resistance models was evaluated at the downstream level of the insulin signalling cascade by assessing the phosphorylation of AKT. AKT protein was phosphorylated when cells were stimulated with 20nM acute insulin in control and 1,25(OH)₂D₃ treated adipocytes, whereas chronic TNF- α treatment (5ng/ml) decreased AKT phosphorylation by 55% ($p < 0.001$). Pre-treatment with 1,25(OH)₂D₃ markedly restored 60% of AKT phosphorylation ($p < 0.05$) when compared with TNF- α treated cells however the expression of the protein was still significantly different from control levels (Figure 5.3.9). Chronic HG/HI (25mM /100nM) also affected AKT phosphorylation by decreasing the protein by 78% ($p < 0.001$) with no effect of 1,25(OH)₂D₃ preincubation (Figure 5.3.9). As AKT mediates insulin induction of glucose uptake and is important for up-regulation of GLUT4 gene expression and recruitment to the cell surface, it was vital to examine the effect of VitD on GLUT4 expression and translocation. In the present study, it was hypothesized that in SGBS human adipocytes, the favourable effect of 1,25(OH)₂D₃ on insulin sensitivity on TNF- α and HG/HI-induced IR cells is mediated by an effect of 1,25(OH)₂D₃ on GLUT4. First, GLUT4 gene and protein expression were measured, then IF visualization in SGBS human adipocytes was established to confirm the effect of 1,25(OH)₂D₃ on GLUT4 translocation. Data revealed that SGBS cells expressed 90% less GLUT4 mRNA ($p < 0.001$) when exposed to TNF- α and this was improved by 70% and 144% ($p > 0.05$) when preincubated with 1,25(OH)₂D₃ of both 10nM and 100nM, respectively. However, although there

was an improvement with $1,25(\text{OH})_2\text{D}_3$ preincubation (not statistically significant), gene expression levels were not restored to control levels. No changes were observed in GLUT4 gene expression when exposed to chronic insulin environment (Figure 5.3.10 (A)). Interestingly, GLUT4 protein expression was significantly upregulated by 1.4-fold when exposed to $1,25(\text{OH})_2\text{D}_3$ ($p < 0.005$), however no changes were observed in total protein when exposed to TNF-alpha. On the other hand, HG/HI treatment resulted in 20% ($p > 0.05$) lower expression of GLUT4 protein and this been decreased and reached significance when preincubated with $1,25(\text{OH})_2\text{D}_3$ ($p < 0.005$) (Figure 5.3.10 (B)). Finally, data from Immunofluorescence confocal imaging of labelled GLUT4 at the plasma membrane showed that GLUT4 fluorescence was attenuated when chronically exposed to TNF-alpha treatment and no response was observed after acute insulin (100nM) stimulation. However, this effect was prevented by $1,25(\text{OH})_2\text{D}_3$ and the translocation of GLUT4 to cell membrane was obvious when preincubated with 100nM of $1,25(\text{OH})_2\text{D}_3$ with TNF-alpha. Chronic HG/HI environment also affected the GLUT4 translocation response and results showed that even with $1,25(\text{OH})_2\text{D}_3$ preincubation, acute insulin induction did not stimulate GLUT4 recruitment to the cell surface (Figure 5.3.11).

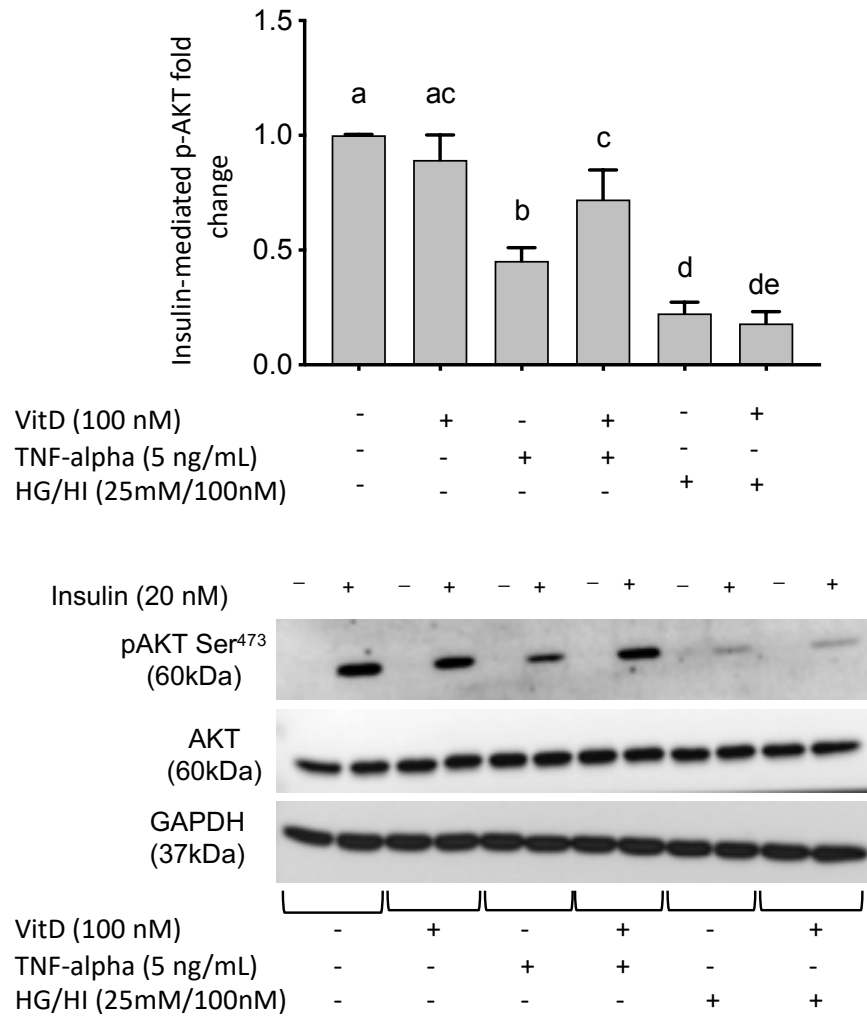


Figure 5.3.9 Effect of 1,25(OH)₂D₃ on AKT phosphorylation in human adipocytes: Representative western blot analysis of three experiments performed in six samples for each treatment showing pAkt Ser473 before and after 10 min of 20 nM insulin stimulation in SGBS adipocytes. (A) bands of p-AKT, total AKT, and GAPDH quantified ratios (mean ± standard error of the mean) of the bands densities of p-AKT by dividing band densities of p-AKT over the total and GAPDH was used as the endogenous control, and (B) the average fold change of phosphorylated AKT level after insulin stimulation relative to controls. in SGBS mature adipocytes preincubated with 1,25(OH)₂D₃ (100 nM) for 24h, followed by chronic TNF-alpha (5ng/ml) or chronic high glucose (25mM) plus high insulin (100nM) (HG/HI) for another 24h. Expression values in control were set at one to assess fold change from this value in other treatments. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$.

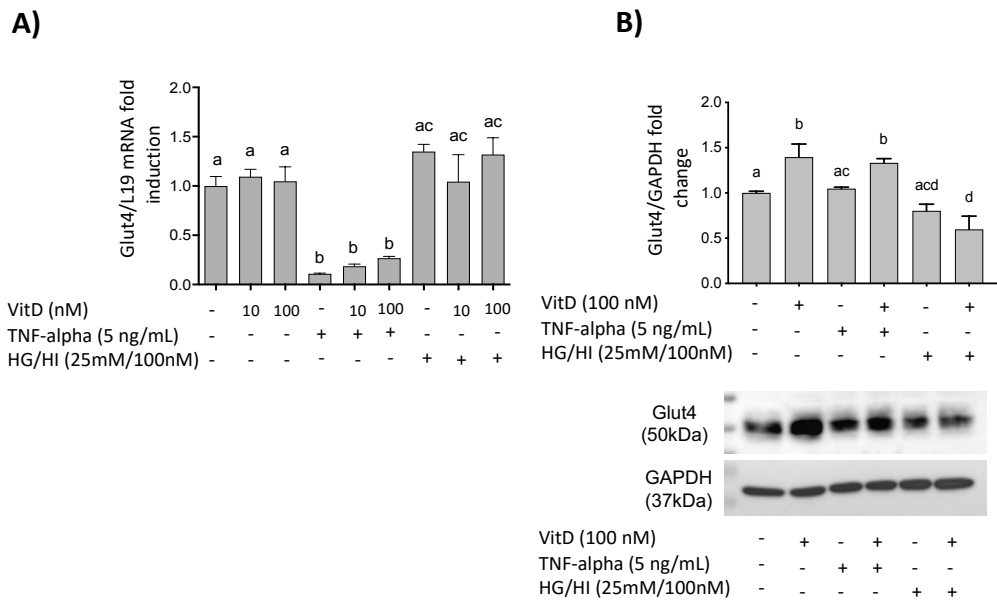
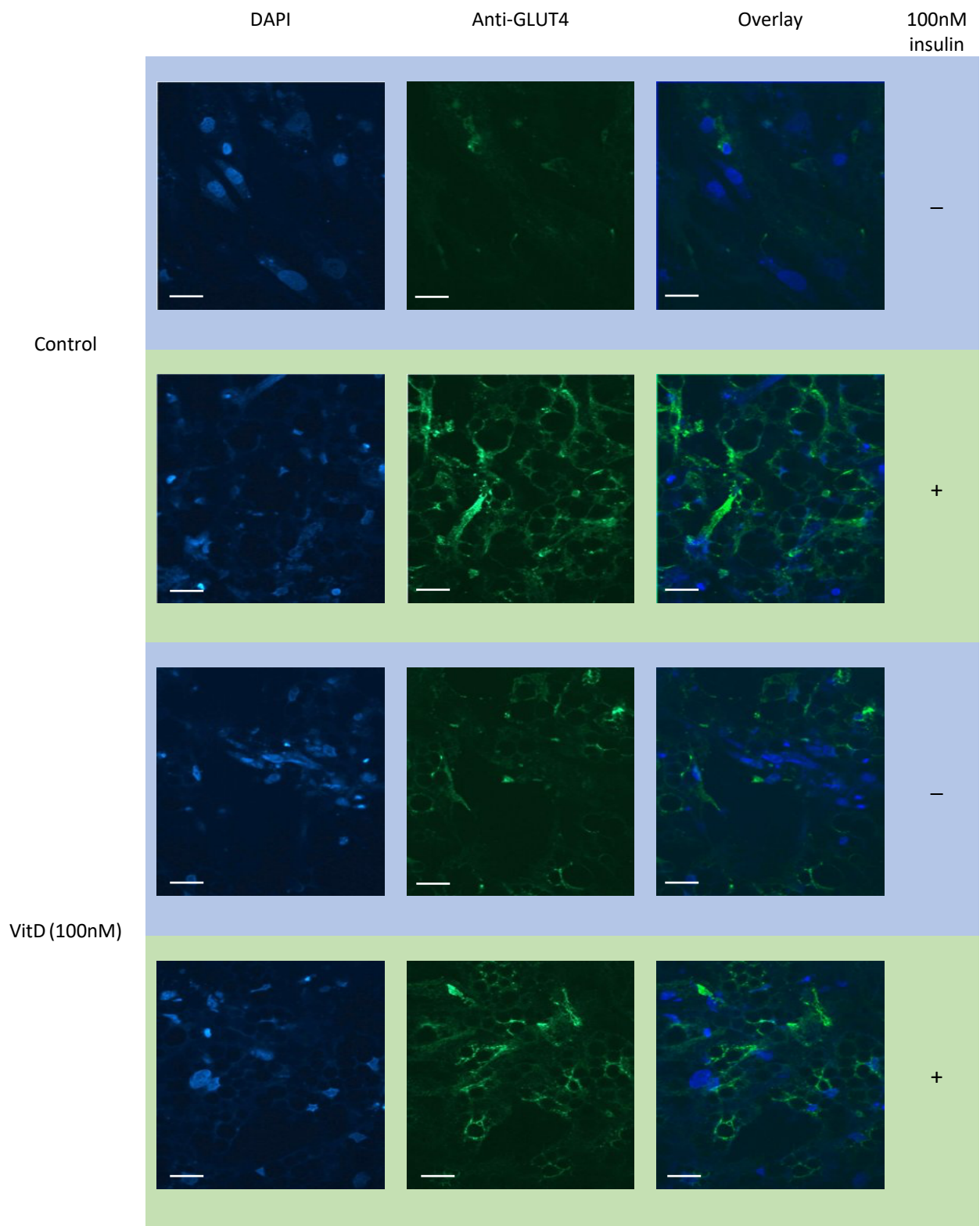
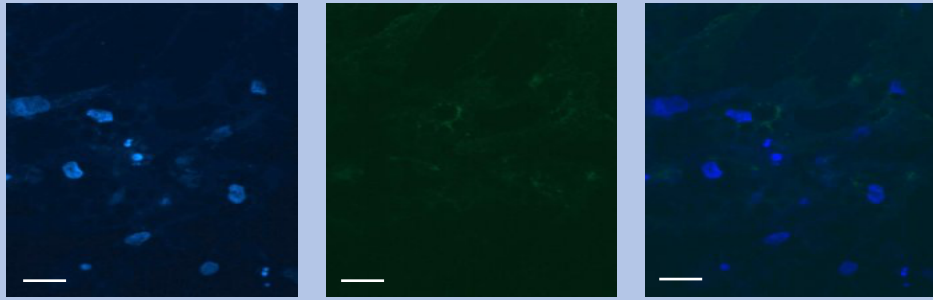


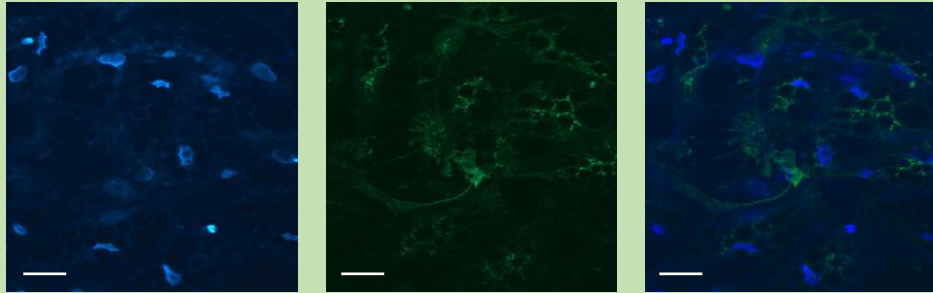
Figure 5.3.10 Effect of 1,25(OH)₂D₃ GLUT4 expression in mature SGBS adipocytes: Mature adipocytes were preincubated with 1,25(OH)₂D₃ (10nM or 100 nM) for 24 h, followed by chronic TNF-alpha (5 ng/ml) or chronic high glucose (25mM) plus high insulin (100nM) (HG/Hi) stimulation for another 24 h. (A) expression levels of GLUT4 mRNA were quantified by qRT-PCR. (B) Representative WB of SGBS mature adipocytes for protein expression and GAPDH was used as the endogenous control. Expression values in control were set at one to assess fold change from this value in other treatments. Bars represent means ± standard error of the mean of three independent experiments done in six samples for each treatment. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test $p < 0.05$



TNF-alpha
(5ng/ml)

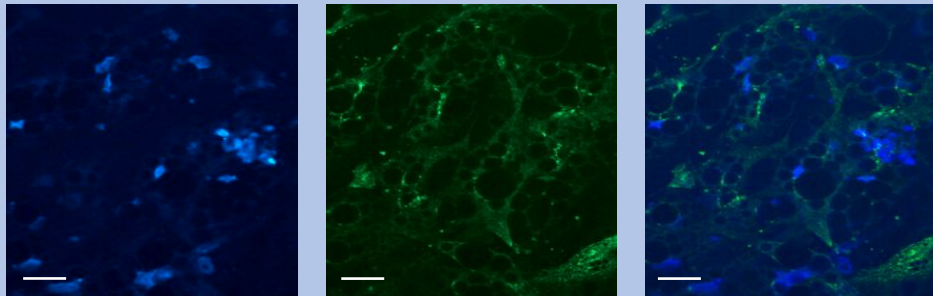


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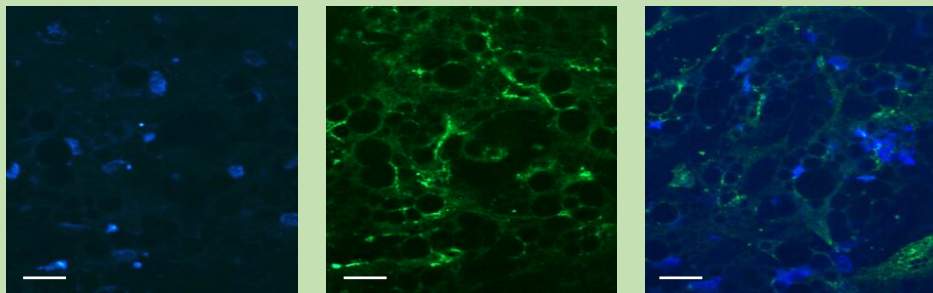


+

VitD (100nM) +
TNF-alpha
(5ng/ml)



-



+

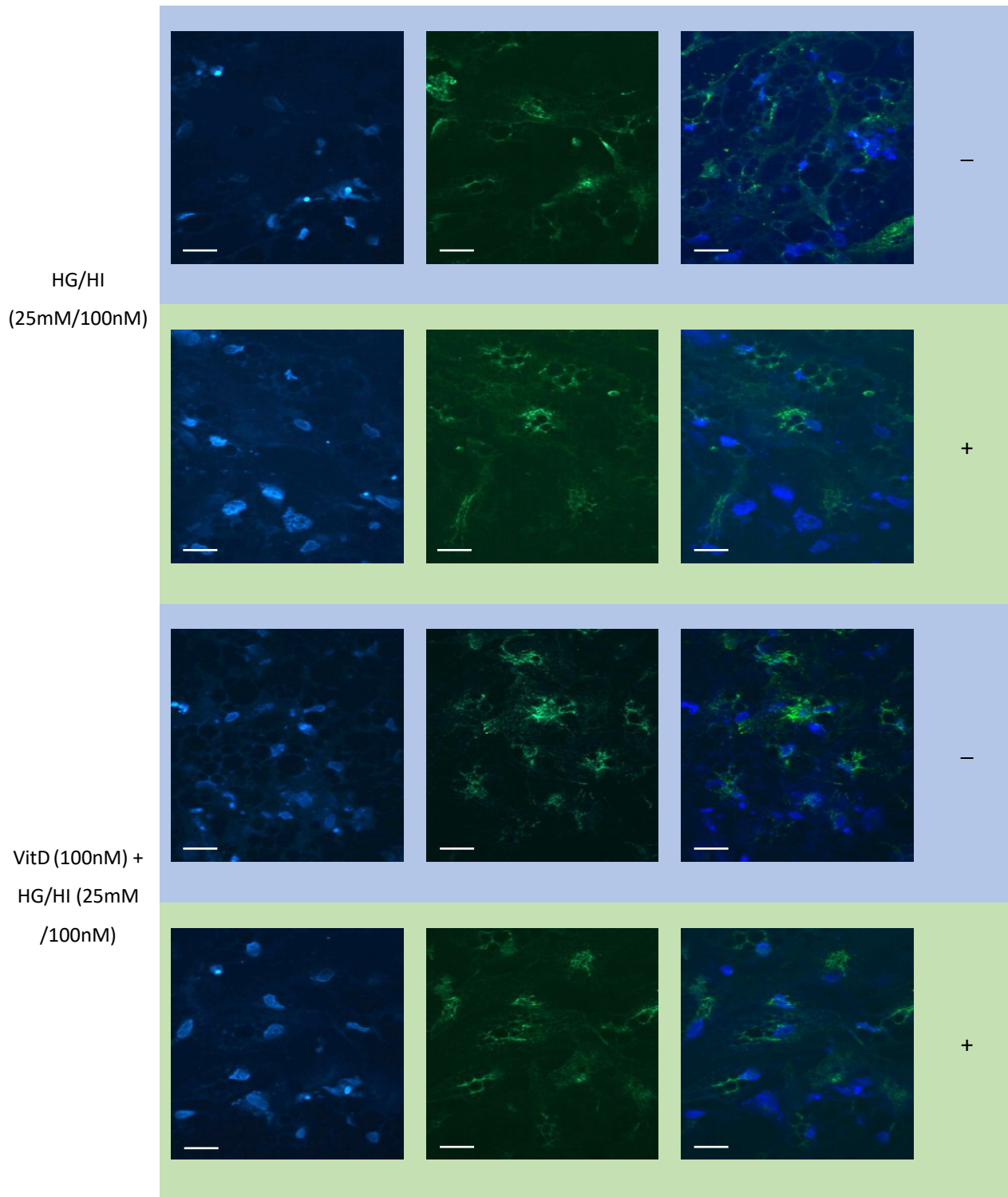
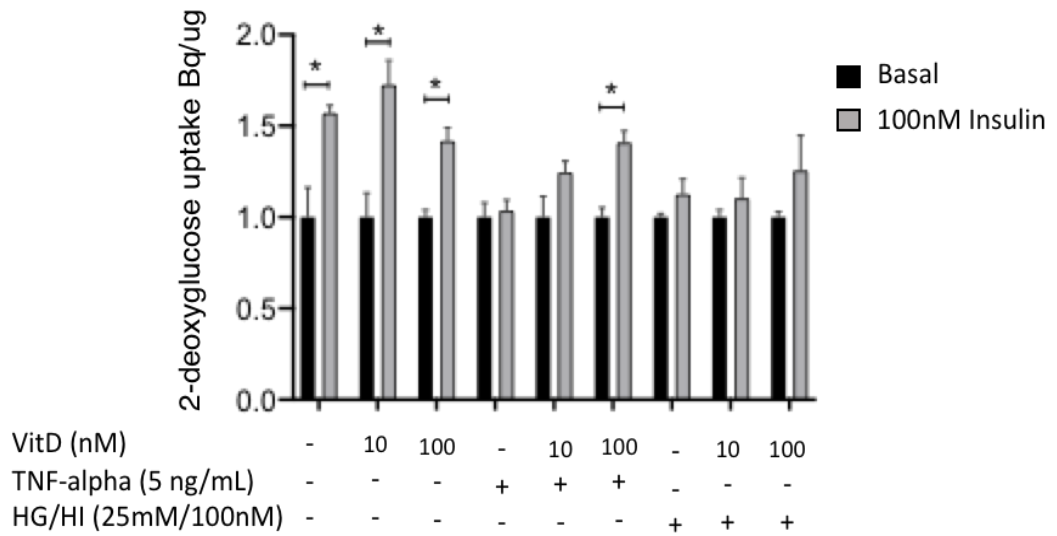


Figure 5.3.11 Confocal Microscopy of GLUT4 translocation in Mature SGBS Adipocytes preincubated with 1,25(OH)₂D₃, followed by TNF-alpha or high insulin treatments: Mature SGBS adipocytes were preincubated with 1,25(OH)₂D₃ (100 nM) for 24h, followed by TNF-alpha (5ng/ml) or high insulin (5ng/ml) for another 24h then each treatment was mediated with 100nM insulin for 15 minutes (green background). Expression and membrane localization of GLUT4 was determined by IF detection using primary and secondary antibodies, anti-GLUT4 and goat anti-rabbit IgG (Alexa Fluor 488), respectively. Nuclei were stained with DAPI, and photomicrographs were obtained using confocal microscope at 40x magnification, scale bar represents 10 μm.

5.3.5 VitD improved 2-deoxyglucose uptake by SGBS cells

To confirm that VitD can indeed stimulate glucose uptake by SGBS adipocytes, a glucose uptake assay been performed. Chronic TNF-alpha treatments (5ng/ml) for 24 hr showed a 36% ($p < 0.005$) reduction in uptake of radio-labelled glucose following an acute dose of insulin (20nM) compared with control adipocytes, whereas glucose uptake was improved by 21% and 37% ($p < 0.05$) when preincubated with 10nM and 100nM of 1,25-(OH)₂D₃, respectively compared with TNF-alpha treated cells (Figure 5.3.12). Although preincubation with 1,25(OH)₂D₃ of both concentrations improved glucose uptake in TNF-alpha treated cells, there was still significant difference in glucose uptake when preincubated with lower concentration of 1,25(OH)₂D₃ ($p < 0.05$) and only the concentration of 100nM restored glucose uptake to control levels. Chronic insulin (HG/Hi) also reduced glucose uptake by 28% ($p < 0.05$) compared with control adipocytes, however, preincubation with 1,25(OH)₂D₃ did not protect against insulin resistance although there was 11.5% improvement in glucose uptake (not significant) when preincubated with 1,25(OH)₂D₃ of 100nM concentration (Figure 5.3.12).

A)



B)

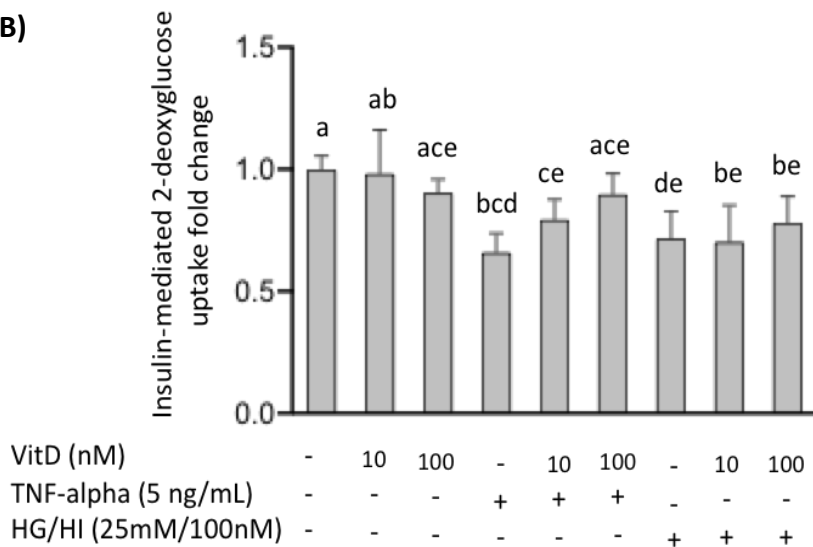


Figure 5.3.12 Effect of 1,25(OH)₂D₃ on human SGBS adipocytes glucose uptake: SGBS adipocytes were preincubated with 1,25(OH)₂D₃ (10 or 100 nM) for 24 hr followed by chronic TNF-alpha (5 ng/ml) or high glucose (25mM) plus high insulin (100nM) (HG/HI) stimulation for another 24 h. (A) 2-deoxyglucose uptake in the basal state (black bars) and after 30 min of 20 nM insulin stimulation (gray bars), average fold change of 2-deoxyglucose uptake is calculated by dividing insulin-mediated over basal uptake for each treatment. Differences between one basal to insulin stimulated levels was determined via 2-tailed paired t-test, *p<0.05. (B) fold changes of insulin-mediated 2-deoxyglucose uptake relative to controls (where only insulin mediated samples were chosen for the comparison). Data are presented as mean ± standard error of the mean of six samples of each treatment. Bars not sharing the same letter were significantly different in Tukey-Kramer post hoc test *p* < 0.05.

5.4 Discussion

The main aim of this study was to investigate the effect of Vit D on inflammation and glucose homeostasis through its involvement in different pathways including MAPK and NF κ B . Therefore, the preventive effects of 1,25(OH)₂D₃ in SGBS human adipocytes on the expression of inflammatory cytokines and glucose uptake was evaluated. The investigations revealed three key findings. Firstly, 1,25(OH)₂D₃ showed a significant role in preventing expression of inflammatory mediators which were induced by TNF-alpha in human SGBS adipocytes. Secondly, 1,25(OH)₂D₃ blocked NF κ B and MAPK signalling which were activated by TNF-alpha and are involved in inflammation and insulin resistance pathways. Finally, 1,25(OH)₂D₃ successfully improved glucose uptake by enhancing AKT phosphorylation and GLUT4 translocation.

To evaluate the impact of 1,25(OH)₂D₃ on the adipocyte inflammatory response, SGBS mature adipocytes were submitted to classical physiological stresses. To which end, TNF-alpha or HG/HI, considered as major mediators for obesity-related inflammation [143, 146, 383, 491], were used to induce a state of low-grade inflammation in SGBS adipocytes. The role of 1,25(OH)₂D₃ in down-regulating chemokine and pro-inflammatory cytokines expression been widely displayed. This study showed that preincubation with 1,25(OH)₂D₃ significantly downregulated the expression of MCP-1, IL-1 β , and IL-8 in TNF-alpha treated cells. These data agree with a previous studies by Marcotorchino J. et al. and Dauletbaev, N., et al. who reported decreases in MCP-1, IL-1 β , and IL-8 when treated with VitD in inflammation stimulated human and 3T3 adipocytes [147,

380]. Moreover, the present study revealed an induction of IL-1 β with chronic HG/HI environment which is aligned with a previous report by Le, K.A., et al. [508] who showed that chronic hyperinsulinemia is associated with stimulation of systemic inflammation. However, data of the current study revealed that when SGBD cells were preincubated with 1,25(OH) $_2$ D $_3$, a significant decrease in the expression of IL-1 β was observed. Taken together, these data suggest that 1,25(OH) $_2$ D $_3$ has a protective effect by restraining the inflammatory response in adipocytes.

The expression levels of the VitD target gene CD14 [509] were also investigated as it has a critical role in pro-inflammatory cytokine production [510]. Current findings showed that these levels were not affected by either chronic TNF-alpha or HG/HI environments which is inconsistent with other studies of different cell types [511-513]. These findings informed the question of to what extent TLR4 is expressed in SGBS cells (since CD14 is a co-receptor for TLR4) [514]. The gene expression data revealed that SGBS cells expressed significantly less TLR4 compared to all cell types tested which might suggest that VitD still works to reduce inflammation suggesting at least in this cell type other mechanisms operate to reduce inflammation. Moreover, sCD14 was found to be elevated in several inflammatory states and is believed to be a potent anti-inflammatory mediator [515, 516]. Data of current study showed a remarkable synergistic increase of CD14 gene expression when 1,25(OH) $_2$ D $_3$ was combined with TNF-alpha or HG/HI treatments as it could be activated by presence of VitD with inflammation which is in agreement with previous studies [509, 517]. Moreover,

the release of sCD14 was significantly increased when inflammation was initiated in the presence of $1,25(\text{OH})_2\text{D}_3$. These findings might suggest the anti-inflammatory effects of $1,25(\text{OH})_2\text{D}_3$ in adipocytes are explained at least in part by their inhibitory action on inflammation through increased release of sCD14. Furthermore, expression of the receptor of VitD and activator of its biological functions, VDR was also investigated. Interestingly, there was a significant increase of VDR gene expression in response to TNF- α treatment which may indicate a compensatory mechanism in response to VitD absence or deficiency as suggested by Wada, K., et al. [460]. In contrast, VDR was significantly reduced when $1,25(\text{OH})_2\text{D}_3$ was present suggesting the activation of D/VDR anti-inflammatory pathway [518].

As the link between adipose tissue inflammation and insulin resistance is mainly through two pathways: NF κ B and MAPKs [334, 499-501] and as activated MAPKs is also able to activate NF κ B [333], the effect of $1,25(\text{OH})_2\text{D}_3$ on NF κ B and protein kinases was examined. Gene and protein expression data revealed that $1,25(\text{OH})_2\text{D}_3$ down-regulated TNF- α -induced NF κ B protein in a dose dependent manner, which suggests that $1,25(\text{OH})_2\text{D}_3$ exerts an inhibitory effect on TNF- α -stimulated cytokine mRNA and protein expression. The results presented are in accord with previous studies that showed deletion of the VDR gene resulted in higher expression of inflammatory genes and less I κ B α protein expression (a potent inhibitor of NF κ B) in different cell models including adipocytes and no change of the expression level of inflammatory genes were observed in the presence of $1,25(\text{OH})_2\text{D}_3$ [147, 519, 520]. In addition to NF κ B

induction by TNF-alpha, present findings showed a sustained phosphorylation of ERK and p38 in SGBS mature adipocytes was observed when stimulated with TNF-alpha. However, preincubation with $1,25(\text{OH})_2\text{D}_3$ significantly inhibited ERK1/2 and p38 phosphorylation and restored them to control levels in human SGBS cells. These data agree with previous data that demonstrated inhibition of p38 and ERK1/2 phosphorylation with $1,25(\text{OH})_2\text{D}_3$ preincubation in different cell types [147, 332]. Current findings suggest that sufficient VitD levels may play a significant role for reducing the inflammatory load in obese individuals and patients with insulin resistance through its inhibitory role of NF κ B production and MAPKs activation.

This study has found that insulin sensitivity was significantly improved with $1,25(\text{OH})_2\text{D}_3$ preincubation in TNF-alpha-induced insulin resistant SGBS adipocytes. The metabolic effects of insulin are first initiated by phosphorylation of its receptor IR. Subsequently phosphorylated IR, stimulates various signalling cascades that eventually stimulate AKT phosphorylation which initiates GLUT4 translocation. Data from this study have demonstrated that $1,25(\text{OH})_2\text{D}_3$ effectively improved AKT phosphorylation which was inhibited by TNF-alpha. As aligned with current findings of Marcotorchino J. *et al* [147] who has shown that $1,25(\text{OH})_2\text{D}_3$ treatment restored AKT phosphorylation in adipocytes. On the other hand, data of this study are not consistent with those of Wang Q. *et al* [521] who reported an inhibition of AKT phosphorylation with $1,25(\text{OH})_2\text{D}_3$ treatment in peritoneal macrophages indicating different roles of VitD in different cell types and indicates further *in vivo* studies are required for more understanding. The

Phosphorylated/activated AKT initiates GLUT4 translocation from the intracellular site to the cell surface to transport glucose into the cell. Present findings have shown that TNF-alpha and HG/HI treatments had noticeably inhibited GLUT4 translocation. However, 1,25(OH)₂D₃ effectively stimulated GLUT4 translocation to cell membrane in TNF-alpha-induced insulin resistant SGBS adipocytes. These findings are in line with the fact that proinflammatory cytokines, TNF-alpha in particular, can affect insulin sensitivity leading to local insulin resistance [146, 385]. Similarly, Manna, P. and his colleague [383] have reported that VitD resulted in significant improvement in insulin signalling via GLUT4 translocation in TNF-alpha-induced insulin resistant 3T3 adipocytes. Finally, to confirm that VitD can indeed stimulate glucose uptake by SGBS adipocytes, a glucose uptake assay was performed. Data showed that chronic TNF-alpha and HG/HI treatments significantly reduced glucose uptake and that 1,25(OH)₂D₃ has considerably restored it in TNF-alpha-induced insulin resistant adipocytes but not with HG/HI-induced insulin resistant cells. On the molecular level, VitD may affect insulin sensitivity through its influence on intracellular calcium [176]. Lower levels of VitD can result in elevated intracellular levels of calcium which impairs postreceptor binding insulin actions, such as the dephosphorylation of insulin regulatable glucose transporter (GLUT4) [522, 523].

Finally, the two models used in this study are capable of inducing insulin resistance by different mechanisms. TNF-alpha induces expression of NF-kB, which dephosphorylates the insulin receptor (IR) and thereby inhibits insulin-evoked signalling events [524]. Chronic hyperinsulinemia desensitizes IRs, thereby

reducing insulin-mediated signalling pathways [524]. The beneficial effects of VitD on expression of inflammatory mediators and insulin signalling were mainly observed with the TNF-alpha-induced insulin resistance model. This could be explained by VitD involvement in inflammatory signalling pathways in which VDR is involved [279]. This assumption could be supported by the observed significant increase of VDR mRNA gene with the TNF-alpha model and the absence of any response towards HG/HI treatment.

In summary, this is the first study to provide data that strongly supports the anti-inflammatory effects of active VitD (1,25(OH)₂D₃) in SGBS adipocytes and its ability to improve the insulin-stimulated glucose uptake through its favourable impact on different pathways including NFκβ and MAPKs signalling. Moreover, this is the first study to show that VitD may exert its favourable effects on improving insulin sensitivity only if insulin resistance was due to increased cytokine production rather than higher doses of insulin. Additional work on the molecular studies of VitD in other cell systems regulating inflammation and insulin sensitivity would further our current insights.

Chapter 6

Discussion and Conclusions

Industrialization and urbanization have caused dramatic changes that has led to obesity pandemic. The abundance of low-cost, high-calorie and nutrient poor foods has caused excessive calorie intake and driven obesity. Despite the excess of dietary calorie consumption, people with obesity have relatively high rates of micronutrient deficiencies. VitD deficiency is considered the most common of these nutrient deficiencies, during expanded fat tissue, due to its fat solubility nature [174]. The importance of VitD as a cofactor in glucose homeostasis and pancreatic β -cell function suggests that deficiency in this micronutrient may play a role in the development of insulin resistance and T2DM particularly in people with obesity [172]. Globally, obesity and VitD deficiency are considered as independent factors for the increased prevalence of T2DM. Approximately 85% of obese people are VitD deficient [174] in which 46% are at higher risk to develop T2DM [447]. Demonstration of a causal role for VitD deficiency in this disorder would lead to new targets for efforts to prevent insulin resistance and T2DM at the population level and, possibly, for its treatment.

Several strategies have been established to treat obesity comorbidities including lifestyle interventions, pharmacotherapy and surgical interventions [340]. Examination of the current literature has been carried out to have a general view about the effect of weight loss on diabetes remission and whether this could be linked to baseline VitD status. This literature review revealed important conclusions. Firstly; very few studies have investigated the link between baseline levels of VitD with T2DM outcomes post significant weight loss [368]. Secondly; regardless of VitD supplementation, some of the studies have demonstrated an

improvement in VitD status after significant fat loss [525] but others showed that the majority of participants still had low levels [368]; which raised the question as to whether VitD metabolism is affected during obesity and whether the changes are due to improvements in VitD metabolism within WAT. Finally, prior *in vitro* investigation of the effects of VitD in AT on inflammation and insulin resistance, informed the need for more molecular studies on adipose tissue to assess the role of VitD in metabolic health by its involvement in different molecular pathways [299, 300, 526, 527]. Therefore, due to these aforementioned conclusions, my studies sought to investigate the effect of VitD on obesity-mediated insulin resistance and whether VitD metabolism is affected by obesity. This would produce favourable treatment strategies for obesity-related diabetes and insulin resistance. The aim of the present thesis was to approach this subject through understanding the mechanisms of VitD treatment to consider favourable treatment strategies.

From the clinical research perspective, the aim of this thesis was to investigate the effect of baseline VitD status on T2DM outcomes post major weight loss on a larger sample size than has been considered in the current literature. The collective evidence to date suggests that the ability of bariatric (metabolic) surgery to improve metabolic syndrome makes it the most effective intervention compared to the non-surgical ones including lifestyle or pharmacotherapy. However, although some bariatric surgery candidates exhibit the same percentage of excess weight loss, bariatric surgery will eventually fail to result in the anticipated resolution of comorbidities [528]. Therefore, several

attempts been taken to improve bariatric surgery outcomes in terms of comorbidity remission based on monitoring presurgical factors such as presurgical weight loss and quitting smoking which were linked to favourable outcomes post-surgery [416]. Few studies have examined the effect of baseline VitD on metabolic outcomes including T2DM. One such study examined 46 patients undergoing gastric bypass and sleeve gastrectomy [368] and demonstrated a significant relationship between levels of baseline VitD and the persistence of T2DM after bariatric surgery (percentage not specified) [368]. The observational prospective study on 309 candidates for sleeve gastrectomy, in this thesis, is the largest study to date that has sought to investigate baseline effects of VitD on different T2DM biomarkers and remission post sleeve gastrectomy. Among subjects who started the surgery pathway with T2DM, no significant differences were found in baseline BMI, EWL and distribution of comorbidities between patients who were in remission and patients who remained with T2DM 1 year post surgery. The findings of this study revealed that half of the participants were in remission and 49% remained with T2DM after the surgery in which VitD explained 27% of the presence of T2DM 1 year post-operatively. Another important finding from this study is that VitD explained 10% of weight regained 18 months post-surgery. However, it was not associated with weight loss post-operatively which points towards the hypothesis that VitD can prevent weight gain, but does not lead to weight loss. The concept that VitD is involved in adipogenesis by inhibiting adipose tissue differentiation might explain the current clinical findings [263, 277, 436]. As higher VitD stored in WAT might cause less differentiation of pre-adipocytes to adipocytes and hence prevent weight gain.

From the basic research perspective, the aim was to investigate whether low circulating VitD levels observed in obesity are due to altered VitD metabolism in AT, and whether major weight loss could lead to improvements in VitD metabolism within AT and hence improvements in VitD circulating levels. Recent studies detail the presence of VDR expression and VitD hydroxylases in subcutaneous and visceral/or omental AT [225, 379, 461]. Higher VitD metabolic components within AT been suggested to be an independent factor for lower circulating VitD as mature adipocytes are capable of taking up VitD from the circulation and converting it to the biologically active form to the adjacent microenvironment [279]. This could explain the lower circulating levels during expanded AT in obesity [412]. The data in this thesis demonstrates for the first time that obese and overweight individuals (who also had lower circulating levels) express significantly higher levels VitD metabolic components within subcutaneous AT which are negatively associated with circulating levels. After weight loss, plasma VitD levels are still below the normal levels and these were associated with higher expression of VitD hydroxylase (CYP27A1) in subcutaneous AT which was also positively associated with fat mass. Within our bariatric cohort, patients' mean BMI was still at obese category 6 months post-surgery which might explain why the majority of patients still had low VitD levels. This might be evident by the observations of VDR gene which did not change 6 months after bariatric surgery. These findings suggest that to be able to reach favourable VitD levels, an individual should reach the lean state for VDR and VitD hydroxylases to decrease within AT leading to higher circulating levels of VitD.

From the molecular research perspective, the aim was to gain greater understanding of the preventive effects of VitD from insulin resistance and inflammation through its involvement in different cellular pathways. In addition to the controversial roles of VitD on adipocyte inflammation and insulin resistance [299, 300, 526, 527], the effect of VitD on inflammation and insulin resistance by looking at multiple pathways and biomarkers have never been published together in a single study. The findings from this study are the first to strongly support the anti-inflammatory effects of VitD in SGBS adipocytes and its ability to improve the insulin-stimulated glucose uptake through its favourable impact on different pathways including AKT phosphorylation, GLUT4 translocation, NF κ B and MAPKs signalling. Moreover, data in this thesis also highlighted, for the first time, that VitD can exert more favourable effects on insulin sensitivity during inflammation than during chronic high insulin levels. These data can explain the rationale of the importance of sufficient VitD levels prior to stressful states such as surgeries.

6.1 Conclusion

The evidence set out in this thesis (and by previous studies), overwhelmingly supports the notion that VitD plays a central regulatory role in human health, and that its levels and metabolism are affected by adiposity. Significant insight has been made in this thesis with our understanding of the physiological mechanisms of favourable roles of VitD in inflammation and obesity-related insulin resistance and T2DM; and how adiposity might affect its levels and metabolism. In particular, the key fact that VitD is involved in multiple regulatory pathways within SGBS adipocytes including NF κ B and MAPKs signalling, AKT phosphorylation and GLUT4 translocation could explain how patients with higher levels of VitD prior to bariatric surgery had an improved chance of remission post-operatively. Moreover, the fact that VitD metabolism is affected during adiposity and is reserved post major weight loss has confirmed at least one reason behind low circulatory levels during obesity. Findings from this thesis suggest the importance to set normal VitD levels as a criteria that patients should meet in order to receive bariatric surgery particularly patients with T2DM. In addition, since VitD levels are associated with AT VitD hydroxylase, which is associated with body weight and fat mass, individuals with greater weight or fat mass may require higher doses of VitD to reach acceptable circulating levels.

6.2 Limitations

There were certain limitations in this thesis that would need to be accounted for in any future work. First is the retrospective approach in chapter 3, as the data on sun light exposure, dietary, physical activity, smoking, was not readily available. In addition, the study would have been complemented if serum 25(OH)D had been collected at different times point prior to the surgery in order to compare the changing circulating levels pre and post-surgery and support to investigate the effect of weight loss on serum level improvements.

In chapter 4, VitD metabolic components were studied from two different cohorts; between lean, overweight and obese individuals and in patients undergoing three different bariatric surgeries. Unfortunately, samples of Om AT were not available from the weight loss trial, which could have provided additional information. Furthermore, an additional limitation in this study was that there was no available data on how much sunshine the participants were exposed to.

6.3 Future work

The work in this thesis has highlighted several areas which it may be beneficial to study in the future. Firstly, despite the aggressive VitD supplementation to achieve normal circulating levels, some SG participants continue to show low levels of VitD. Therefore, further studies are needed with more details on VitD levels before and after VitD supplementation prior to the surgery to establish an algorithm for optimal pre-surgical doses.

Secondly, longitudinal pre and post major weight loss studies that only include patients who reach normal body weight and fat mass post weight loss may be beneficial to further investigate the changes in VDR and VitD metabolic components and their association with circulating VitD levels. Moreover, it will be beneficial to measure the levels of $1,25(\text{OH})_2\text{D}_3$ (the active form) in AT and investigate its association with VitD metabolic components within the same environment in order to confirm that AT is truly able to metabolise VitD. Moreover, there is potential for *in vivo* studies on the effect of VitD treatment on the gene expression levels of VitD hydroxylases in AT which would help to consider VitD supplementation for treatments of impaired VitD metabolism in AT for patients with obesity. It is also important to note that the molecular mechanisms involved in VitD uptake and secretion by AT have not yet been investigated and looking at this area will add valuable findings to the literature.

Finally, more studies on human primary adipocytes from different BMIs, ages or metabolic conditions are needed to gain greater understanding and validation about the involvement of VitD on inflammatory and insulin signalling

pathways. In addition, there is the potential to explore the effect of VitD of different doses/time series on adipogenesis in order to gain better understanding on its role in managing AT expansion.

Appendix 1: WISDEM Staff

Warwickshire Institute for the Study of Diabetes, Endocrinology and Metabolism
Staff (2011-2018):

Prof. Harpal Randeva	(WISDEM Clinical Director, Consultant Endocrinologist)
Mr. Vinod Menon	(Consultant Surgeon)
Mr. FT Lam	(Divisional Medical Director)
Mr. Ian Fraser	(Consultant Surgeon)
Mr. Matthew Venus	(Consultant Plastic Surgeon)
Mrs. Jenny Abraham	(Specialist Bariatric Nurse)
Ms. Lisa Attenborrow	(Bariatric Coordinator)
Prof. Sudhesh Kumar	(Professor of Medicine, Diabetes and Metabolism)
Dr. Tom Barber	(Associate Professor of Endocrinology)
Prof. Grigorios Kaltsas	(Consultant Endocrinologist)
Dr. Milan Piya	(Consultant Endocrinologist)
Dr. Hassan Kahal	(NIHR Clinical Lecturer)
Dr. Hema Venkataraman	(ST3 Endocrinology and Diabetes)
Dr. Daniel Border	(ST2 Acute Internal Medicine)
Dr. Georgios Dimitriadis	(Clinical Research Fellow)
Dr. Helen Miller	(Clinical Psychologist)
Dr. David Kendrick	(Clinical Psychologist)
Dr. Emma Shuttlewood	(Bariatric Clinical Psychologist)
Miss Neha Shah	(Specialist Bariatric Dietitian)
Mrs. Louise Halder	(Senior Bariatric Dietitian)
Mrs. Olga Sutton	(Senior Dietitian)
Ms Wendy Clayton	(Diabetes Specialist Nurse)
Ms Wendy Goodwin	(Diabetes Specialist Nurse)
Dr. Narendra Reddy	(NIHR Clinical Lecturer)
Dr. Saboor Aftab	(NIHR Clinical Lecturer)
Dr. Umar Shariff	(Registrar)
Dr. Aruna Munasinghe	(Clinical Fellow)
Dr. Shameen Jaunoo	(Clinical Fellow)
Dr. Peng Cheun Lau	(Clinical Fellow)

Appendix 2: List of Formal Presentations during PhD Studies (2016-2019)

Dates May 3rd - 5th, 2017
Conference ASGBI International Surgical Congress
Venue SCOTTISH EXHIBITION AND CONFERENCE CENTRE, GLASGOW
Presentation Pre-surgical predictors of post-surgical weight loss: role of dietetic follow-up and patient engagement (POSTER)

Dates May 3rd - 5th, 2017
Conference ASGBI International Surgical Congress
Venue SCOTTISH EXHIBITION AND CONFERENCE CENTRE, GLASGOW
Presentation Is pre-surgical weight loss a good predictor of post-surgical metabolic outcomes? (**ORAL**)

Dates Aug 29th - Sep 2nd, 2017
Conference 22nd World Congress - International Federation For The Surgery Of Obesity and Metabolic Disorders – IFSO2017
Venue QEII Centre, London
Presentation Is pre-surgical weight loss a good predictor of post-surgical metabolic outcomes? (POSTER)

Dates Aug 29th - Sep 2nd, 2017
Conference 22nd World Congress - International Federation For The Surgery Of Obesity and Metabolic Disorders – IFSO2017
Venue QEII Centre, London
Presentation Pre-surgical predictors of post-surgical weight loss: role of dietetic follow-up and patient engagement (POSTER)

Dates Jun 9th – 13th, 2017
Conference American Diabetes Association 77th Annual Scientific Sessions
Venue San Diego, CA, USA
Presentation Can baseline Vitamin D levels predict type 2 diabetes remission following sleeve gastrectomy? (**ORAL**)

Dates Jun 22nd – 26th, 2018
Conference American Diabetes Association 78th Annual Scientific Sessions
Venue Orlando, FL, USA
Presentation Baseline Vitamin D as a surrogate marker for type 2 diabetes remission and weight loss following sleeve gastrectomy (POSTER)
Diabetes 2018 Jul; 67(Supplement 1):
<https://doi.org/10.2337/db18-786-P>

Dates Dec 6th – 7th , 2018
Conference 44th Adipose Biology from Conception to Death - ATDG
Venue The University of Edinburgh, Edinburgh
Presentation Can Baseline Vitamin D Used as a predictor for Type 2 Diabetes Remission and Weight Loss Post Laparoscopic Sleeve Gastrectomy? (POSTER)

Dates Jan 24th – 25th , 2019
Conference The Europa Hotel, Belfast
Venue 10th BOMSS Annual Scientific Meeting
Presentation Successful T2DM Remission post Sleeve Gastrectomy is Dependent on Higher Baseline Vitamin D Levels (POSTER)
Obesity Surgery (2019) 29 (Suppl 1):S18
<https://doi.org/10.1007/s11695-019-03786-8>

Dates Mar 19th , 2019
Conference Bariatric Surgery Service Away Day
Venue Saxon Mil, Coventry
Presentation Can Baseline Vitamin D Levels Predict weight loss and T2DM remission following SG? (**ORAL**)

Appendix 3: Changes in clinical and biological characteristics from baseline to 6 months and 1 year post surgery in non-T2DM

Comparison between biological characteristics in non-T2DM patients at baseline, 6 and 12 months after SG

	Baseline			6 months			12 months		
	Mean±SD (n)	Min-Max	Mean±SD (n)	Min-Max	Mean±SD (n)	Min-Max	Mean±SD (n)	Min-Max	
Vitamin D (nmol/l)	43.47±22.77 (123)	10-113	73.54±25.3 (134)a	10-145	75.45±25.47 (139)c	10-145	75.45±25.47 (139)c	16-137	
FPG (mmol/l)	4.85±0.67 (157)	3.5-7.6	4.72±0.8 (113)	2.8-8.5	4.59±0.7 (122)c	2.8-8.5	4.59±0.7 (122)c	3.5-7.8	
HbA1c (mmol/mol)	38.09±4 (147)	25-47	NA	NA	34.28±3.87 (139)c	NA	34.28±3.87 (139)c	23-43	
Cholesterol (mmol/l)	5.13±0.96 (145)	2.5-7.7	4.89±0.95 (107)ab	2.5-7.4	5.03±0.95 (121)	2.5-7.4	5.03±0.95 (121)	2.5-7.7	
HDL-Chol (mmol/l)	1.33±0.32 (145)	0.6-2.2	1.44±0.44 (107)ab	0.7-4.1	1.66±0.38 (121)c	0.7-4.1	1.66±0.38 (121)c	0.9-2.5	
TG (mmol/l)	1.51±0.71 (129)	0.6-5	1.34±0.8 (103)ab	0.5-7	1.16±0.53 (108)	0.5-7	1.16±0.53 (108)	0.4-4.2	
Chol:HDL	4.02±1 (145)	1.9-7.8	3.61±1.1 (107)ab	1-8.1	3.15±0.87 (121)c	1-8.1	3.15±0.87 (121)c	1.7-6.8	
LDL-Chol (mmol/l)	3.11±0.86 (129)	0.8-5.4	2.88±0.85 (103)a	1.1±4.9	2.86±0.89 (108)c	1.1±4.9	2.86±0.89 (108)c	0.8-5	
AST (U/l)	77.25±21.91 (163)	36-240	74.7±30 (133)a	40-281	70±22.54 (139)c	40-281	70±22.54 (139)c	36-190	
ALT (U/l)	23.28±13.66 (162)	5-91	15.91±15.32 (133)a	5-170	13.47±5.57 (139)c	5-170	13.47±5.57 (139)c	5-37	
CRP (mg/l)	12.1±9.7 (57)	3-44	NA	-	NA	-	NA	-	
*CRP+1 (mg/l)	30.65±32.69 (131)	3-272	NA	-	NA	-	NA	-	
T4 (ug/dl)	15.74±3.98 (74)	8-33	NA	-	15.82±4.2 (41)	-	15.82±4.2 (41)	2.6-24	
TSH (mIU/l)	2.37±2 (144)	0.02-18	NA	-	2.2±4.11 (90)	-	2.2±4.11 (90)	0.02-39	
Systolic BP (mmHg)	140.11±17.2 (106)	104-190	NA	-	129.88±19.1(89)c	-	129.88±19.1(89)c	84-191	
Diastolic BP (mmHg)	76.29±10.47 (106)	52-103	NA	-	74.65±10.41(89)	-	74.65±10.41(89)	50-104	

*CRP+1 is the level of CRP one day after the surgery. FPG is fasting plasma glucose, TG is triglycerides. a is mean significant difference between baseline and 6 months. b is mean significant difference between 6 and 12 months. c is mean significant difference between baseline and 12 months. Significance level is p<0.05.

Appendix 4: Changes in clinical and biological characteristics from baseline to 6 months and 1 year post surgery in T2DM

Comparison between biological characteristics in T2DM patients at baseline, 6 and 12 months after SG

	Baseline			6 months			12 months		
	Mean±SD (n)	Min-Max	Mean±SD (n)	Min-Max	Mean±SD (n)	Min-Max	Mean±SD (n)	Min-Max	
Vitamin D (nmol/l)	39.96±21 (106)	10-97	69.43±26.28 (95)a	10-126	68.66±25.6 (114)c	15-142			
FPG (mmol/l)	7.91±4.39 (146)	4.2-28.7	5.92±2.41 (97)a	3.4-16.3	5.84±2.37 (107)c	3.6-18.5			
HbA1c (mmol/mol)	58.59±18.87 (143)	31-118	NA	NA	43.59±12.85 (132)c	27-114			
Cholesterol (mmol/l)	4.35±0.95 (136)	1.9-7.4	4.3±0.95 (97)b	2.6-7.1	4.6±1 (113)c	2.7-8.1			
HDL-Chol (mmol/l)	1.15±0.33 (136)	0.6-2.4	1.35±0.45 (97)ab	0.7-3.6	1.45±0.4 (113)c	0.7-3.1			
TG (mmol/l)	1.86±0.9 (115)	0.5-6.1	1.35±0.54 (88)ab	0.5-3.7	1.59±0.76 (93)c	0.4-5.1			
Chol:HDL	4±1.25 (136)	0.4-7.7	3.39±1 (97)a	1.5-6.5	3.37±1.1 (113)c	1.5-6.1			
LDL-Chol (mmol/l)	2.29±0.97 (118)	0.1-5.3	2.3±0.86 (88)	1-4.6	2.45±0.95 (93)	0.9-5.6			
AST (U/l)	80.59±25.46 (145)	37-231	68.27±16.48 (113)a	30-116	72.89±21.23 (126)c	32-173			
ALT (U/l)	28.51±18.87 (144)	7-131	18.49±16 (112)ab	4-161	15.14±7 (126)c	6-48			
CRP (mg/l)	10.88±12.8 (49)	3-63	NA	-	NA	-			
*CRP+1 (mg/l)	26.91±22.7 (122)	3-138	NA	-	NA	-			
T4 (ug/dl)	16.24±5.46 (59)	9-41	NA	-	16.22±4.5 (47)	0.9-31			
TSH (mIU/l)	2.23±1.7 (131)	0.02-11	NA	-	1.57±1.1 (91)c	0.02-5.6			
Systolic BP (mmHg)	142±18.74 (109)	100-194	NA	-	131.8±18.44 (86)c	90-202			
Diastolic BP (mmHg)	76.94±10.95 (107)	48-107	NA	-	75±10.75 (86)	51-103			

*CRP+1 is the level of CRP one day after the surgery. FPG is fasting plasma glucose, TG is triglycerides. a is mean significant difference between baseline and 6 months. b is mean significant difference between 6 and 12 months. c is mean significant difference between baseline and 12 months. Significance level is p<0.05.

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