The Metabolic Effects of Obesity and Bariatric Surgery: Macrovascular Disease Risk, Microvascular Disease and Bone Health Factors

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Abstract

Much of the morbidity and mortality associated with obesity is related to its metabolic complications. Obese individuals are at higher risk of microvascular disease and premature cardiovascular disease (CVD). Bariatric surgery is currently accepted as the most effective treatment for obesity and related co-morbidities. It reduces the incidence of CVD in a weight-independent manner conferring beneficial mechanisms that have remained elusive. However, bariatric surgery has also been associated with nutritional deficiencies which can cause metabolic disturbance, such that observed deteriorations in bone health have been linked to post-operative vitamin D deficiency.

This thesis examined the association between different metabolic factors that are clustered in obesity to elucidate how CVD risk is increased and subsequently reduced following bariatric surgery. There were, in the studies presented, reductions in insulin resistance, inflammatory cytokines, atherogenic lipoproteins and dysglycaemia following bariatric surgery. Consequently, this thesis demonstrated the use of bariatric surgery as a model to further understand the basis of the metabolic syndrome (Reaven's hypothesis), provided evidence of how the metabolic syndrome augments CVD risk and showed that the risk factors are reduced following bariatric surgery.

Studies in this thesis found post-bariatric-surgical improvements in high-density lipoprotein functionality (in a multi-faceted manner) and levels of autoantibodies to apolipoprotein A-1, both of which, in recent years, have been associated with enhanced CVD risk. The increases in HDL function were related to reductions in inflammation following bariatric surgery. Furthermore, this thesis quantitatively demonstrated reductions in diabetic neuropathy (using corneal confocal microscopy) and diabetic kidney disease but not diabetic retinopathy after bariatric surgery. Additionally, sexual dysfunction in obese men was shown to be related to microvascular disease and not hormonal dysfunction; further evidence of the adverse metabolic environment in obesity. Moreover, this thesis highlighted the prevention of vitamin D deficiency following bariatric surgery by using a carefully implemented post-operative programme of nutritional supplementation.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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CONTRIBUTION

Safwaan Adam, the author of this thesis, was actively involved in and made a significant contribution to all of the chapters/studies presented and discussed in this thesis. He co-authored the relevant study protocols for the research presented in this thesis. He also submitted the protocols for ethical review and presented relevant information to the ethical review board. The author recruited participants and oversaw an informed consent process for the study participants. He also performed relevant clinical assessments, neuropathy assessments, administration of questionnaires and venepuncture for blood samples. In addition, he assisted during gluteal subcutaneous adipose tissue biopsies. He separated samples for storage and kept a central data log of sample identities. The author performed selected biochemical tests (interleukin-6 and tumour necrosis factor-α using the ELISA method) under the supervision of Dr Yifen Liu. He also co-ordinated the delivery of samples to relevant collaborators. He acquired retinal images from the NHS Diabetic Eye Screening Programme for independent analysis by consultant ophthalmologists. The author performed statistical analysis for the results presented in this thesis.

Other members of the research team performed the following tasks:

- Patient recruitment and co-ordination was also done by Drs Jan Ho and Shazli Azmi;
- Clinical measurements and neuropathy assessments were also carried out by Drs Jan Ho, Shazli Azmi and Shaishav Dhage;
- Venepuncture was also performed by Drs Jan Ho and Shaishav Dhage;
- Sample separation and processing for storage was also undertaken by Drs Jan Ho and Shaishav Dhage;
- Electrodiagnostic studies were undertaken by Dr Andrew Marshall, consultant neurophysiologist;
- Ophthalmic examinations were performed by Dr Maryam Ferdousi and Miss Alise Kalteniece;
- Gluteal subcutaneous adipose tissue biopsies were undertaken by Dr Reza Aghamohammedzadeh;

- Gluteal adipose tissue biopsy histological analysis was undertaken by Dr Maria Jeziorska;
- Targeted gene expression in adipose tissue biopsies was carried out by Dr Kirk Siddals;
- Biochemistry measurements presented in this thesis were principally done by Dr Yifen Liu with important contributions by Drs Tarza Siahmansur and Jan Ho. Haematology and selected immunology tests (these were done to assess for exclusion criteria and therefore results are not presented) were done by the laboratory medicine department at Manchester University NHS Foundation Trust. Selected biochemistry analyses were performed and reported by the laboratory medicine departments at Salford Royal NHS Foundation Trust (glycated haemoglobin, serum creatinine, bone profile and vitamin D) and Manchester University NHS Foundation Trust (serum creatinine, urinary albumin and creatinine, glycated haemoglobin, androgen profile). Antibodies to apolipoprotein A-1 measurements were carried out by Dr Sabrina Pagano and others in the laboratory of Prof. Nicolas Vuilleumier.
- Analysis of retinal images presented in Chapter 7 were carried out by Drs Yvonne D'Souza and Salim Natha.
- Selected data analysis was also done by Drs Jan Ho (chapter 8), Alistair Fox (chapter 9) and Dr Akheel Syed (chapter 9).

ALTERNATIVE THESIS FORMAT

The author, working in the University of Manchester, Faculty of Biology, Medicine and Health, has been granted permission by his supervisors Dr Handrean Soran, Dr Rachelle Donn and Dr Adam Stevens to submit this Ph.D. thesis in an alternative format under the regulations, including sections which are in a format suitable for submission for publication or dissemination. The following chapters in this thesis that have been published, are under review or are being submitted for publication:

- **Chapter 1.4**: Published in Diabetes and Vascular Disease Research, April 2019

- **Chapter 4**: Published in Diabetes and Vascular Disease Research, April 2019.

- **Chapter 5:** To be submitted for publication

- **Chapter 6:** To be submitted for publication

- **Chapter 7:** Currently under review

- **Chapter 8:** Published in PLoS ONE, September 2019.

- **Chapter 9:** Accepted for publication in Obesity Surgery, September 2019.

List of Abbreviations

-C	Cholesterol
%EBMIL	Percentage Excess Body Mass Index Loss
%TWL	Percentage Total Weight Loss
25(OH)D	25 Hydroxy Vitamin D_3
95% CI	95% Confidence Interval
ABC-	ATP binding cassette
ACE	Angiotensin Converting Enzyme inhibitors
ACS	Acute Coronary Syndromes
AH	Arterial Hypertension
AIM-HIGH	Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
Anti-apoA1-IgG	Autoantibodies against apoA-1
Apo-	Apolipoprotein
ARB	Angiotensin 2 Receptor Blockers
BMD	Bone Mineral Density
BMI	Body Mass Index
BP	Blood Pressure
BPD	Bilio-Pancreatic Diversion
BSA	Bovine Serum Albumin
CCM	Corneal Confocal Microscopy
CEC	Cholesterol Efflux Capacity

CETP	Cholesteryl Ester Transfer Protein
CHOD-PAP	Cholesterol Oxidase Phenol 4-Aminoantipyrine Peroxidase
CIP	Cold Induced Pain
CKD	Chronic Kidney Disease
CKD-EPI$_{cyst\text{-}creat}$	Chronic Kidney Disease Epidemiology Collaboration 2012 equation combining both creatinine and cystatin C
CNBD	Corneal Nerve Branch Density
CNFD	Corneal Nerve Fibre Density
CNFL	Corneal Nerve Fibre Length
CRP	C-Reactive Protein
CS	Cold Sensation
CV	Co-efficient of Variance
CVD	Cardiovascular Disease
CXCL5	CXC Ligand 5
DESP	Diabetic Eye Screening Programme
DiRECT	Diabetes Remission Clinical Trial
DJB	Duodeno-Jejunal Bypass
DKD	Diabetic Kidney Disease
DN	Diabetic Neuropathy
DR	Diabetic Retinopathy
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme Linked Immunosorbent Assay
EMAS-SFQ	European Male Ageing Study Sexual Function Questionnaire

FG	Fasting Glucose
FXR	Farnesoid X Receptor
GBD	Global Burden of Disease
GFR	Glomerular Filtration Rate
GIP	Glucose-dependant Insulinotropic Peptide
GJ	Gastro-jejunostomy
GLP-1	Glucagon Like Peptide-1
GOD-PAP	Glucose Oxidase and 4-Aminoantipyrine
GPO-PAP	Glycerol Phosphate Oxidase Phenol 4-Aminoantipyrine Peroxidase
HbA1c	Glycated Haemoglobin
HDL	High-Density Lipoprotein
HIM Study	Hypogonadism in Males
HOMA-IR	Homeostatic Model of Assessment for Insulin Resistance
HPS2-THRIVE	Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events
HR	Hazard Ratio
hsCRP	high-sensitivity C-Reactive Protein
IL-6	Interleukin-6
IT	Ileal Transposition
LA	Left Atrium
LAGB	Laparoscopic Adjustable Gastric Band
LCAT	Lecithin-Cholesterol Acyltransferase
LDL	Low-Density Lipoprotein
LPS	Lipopolysaccharide

LSG	Laparoscopic Sleeve Gastrectomy
LV	Left Ventricle
mGFR	measured Glomerular Filtration Rate
MPO	Myeloperoxidase
NCS	Nerve Conduction Studies
NDS	Neuropathy Disability Score
NHS	National Health Service
NICE	National Institue for Health and Care Excellence
NIHR	National Institute of Health Research
NSP	Neuropathy Symptom Profile
OD	Optical Density
OR	Odds Ratio
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffer Solution
PCSK9	Proprotein Convertase Subtilisin/kexin type 9
PH	Pulmonary Hypertension
PON1	Paraoxonase-1
PPAR	Peroxisome Proliferator Activator Receptor
PTH	Parathyroid Hormone
PYY	Peptide YY
QST	Quantitative Sensory Testing
RAS	Renin-Angiotensin-Aldosterone System
RBP-4	Retinol-Binding Protein 4
RCT	Reverse Cholesterol Transport

REVEAL	Randomised Evaluation of the Effects of Anacetrapib through Lipid Modification
RV	Right Ventricle
RYGB	Roux-en-Y Gastric Bypass
SAT	Subcutaneous Adipose Tissue
sCreat	Serum Creatinine
sCysC	Serum Cystatin C
SD	Standard Deviation
sdLDL (SD-LDL)	small, dense Low-Density Lipoprotein
SFN	Small Fibre Neuropathy
SHBG	Sex Hormone Binding Globulin
SNS	Sympathetic Nervous System
SOS	Swedish Obese Subjects
SPK	Simultaneous Pancreas and Kidney transplantation
SR-B1	Scavenger Receptor B-1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TNF-α	Tumour Necrosis Factor alpha
uACR	urinary-Albumin-to-Creatinine Ratio
UKPDS	United Kingdom Prospective Diabetes Study
VLDL	Very Low-Density Lipoprotein
VPT	Vibration Perception Threshold
WHO	World Health Organisation
WIP	Warm Induced Pain

WS	Warm Sensation
α	Alpha
Δ	Delta (representing percentage change)

“*The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes.*”

Ibn Sīnā

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I have met some wonderful people in the research group. Dr Jan Ho provided me with intellectual discussion and assisted me with eagerness whenever I needed it. Dr Shaishav Dhage was always keen to help and would make sure I looked after myself. Dr Yifen Liu has been a great teacher and colleague; she has never complained even when she has had to go 'above and beyond'. Dr Maryam Ferdousi and Alise Kalteniece for their helpful discussions and support of my studies when needed. Professor Rayaz Malik has provided me with invaluable advice and a unique perspective on the different research studies.

To my parents, Ebrahim and Ayesha, I cannot express how much I owe them: the support, love, understanding and goodwill they have offered me has allowed me to pursue my career opportunities. Their sacrifice on my behalf is something that I hope the Almighty rewards them for abundantly. I also am eternally grateful to my two brothers Asif and Zulfiquar who have supported me, guided me and reminded me of the importance of dedication, diligence and endeavouring for excellence. This is an opportune moment to also acknowledge the guidance, kindness and encouragement Farouk and Kay Moosa have continually provided me with during all of my studies.

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Finally, this work would not have been possible had it not been for the wonderful volunteers in my research studies. From interacting with them I have learnt to appreciate that the success of my research was wholly dependent on their sacrifice, good-nature and enthusiasm.

Preface

Safwaan Adam underwent his undergraduate medical training at the University of Cape Town and graduated with an MBChB degree in 2005. Subsequently, he did junior doctor rotations in Johannesburg, South Africa where he was presented with the 'Intern of the Year' award (amongst 200 intern doctors). He then continued junior doctor roles in Palmerston North, New Zealand and Bolton, England before starting his core medical training in Manchester in 2009. He was awarded his MRCP in 2010. In 2011, he gained a National Training Number in Endocrinology and completed his Speciality Certificate Examination in endocrinology in 2015. Between 2015 and 2018, he spent 3 years out of postgraduate medical training to undertake research leading up to this thesis. He has finished his speciality training in endocrinology and is now a consultant endocrinologist having been awarded his Certificate of Completion of Training in April 2019.

He has always been keen to pursue an academic career in medicine resulting in research projects and peer-reviewed publications from early on in his medical training. Whilst completing rotations as a speciality trainee, he spent time working in obesity and bariatric clinics which piqued his interest and led to his research in the field.

During his research period, he has published 22 peer-reviewed publications and presented numerous aspects of his work at local, regional, national and international conferences. He achieved first prize for the best oral communication at the North West Endocrine Society annual meeting (2016), the British Obesity and Metabolic Surgery Society annual conference (2018) and the Association of British Clinical Diabetologists annual SpR meeting (2018). He was granted the prestigious Samuel Leonard Simpson Fellowship in endocrinology by the Royal College of Physicians in 2017. He was also awarded an outstanding abstract award by the Endocrine Society for the Annual ENDO meeting in New Orleans (2019). In this meeting he was nominated for the President's Prize.

He has been invited to speak or chair sessions at prestigious meetings including the inaugural Diabetes Professional Care Conference (2015), Royal Society of Medicine (2017), Society for Endocrinology BES annual meeting (2017), HEARTUK annual conference (2018), North West Lipid Forum (2018) and the Royal College of Physicians obesity update (2019).

He actively teaches and supervises medical students and junior doctors having also been the trainee representative for endocrinology trainees in the North West of England.

List of publications during period of registration:

1. Ho JH, **Adam S**, Azmi S, Ferdousi M, Liu Y, *et al.* (2019) Male sexual dysfunction in obesity: The role of sex hormones and small fibre neuropathy. PLOS ONE 14(9): e0221992. https://doi.org/10.1371/journal.pone.0221992

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2. Ho JH, Liu Y, **Adam S**, Azmi S, Dhage SS, Syed AA, Ammori BJ, Donn R, Malik RA, Yang X, Tsimikas S, Soran H.The Effect Of Metabolic Surgery On Lipoprotein (A), Oxidised Phospholipids And Biomarkers Of Lipoprotein Oxidation. (Oral Presentation, HEARTUK Annual Conference, Warwick, 2019)

3. Liu Y, Dhage S, France F, **Adam S**, Ho JH, Donn R, Durrington P, Soran H. Variation And Distribution Of Apolipoprotein E And Its Glycation In Plasma Of Type 2 Diabetes. (Poster Presentation at EAS Annual Meeting, Maastricht, 2019)

4. Ho JH, Liu Y, **Adam S**, Azmi S, Dhage SS, Syed AA, Ammori BJ, Donn R, Gibson MJ, Malik RA, Yang X, Durrington PN, Tsimikas S, Soran H. Divergent Changes In Lipoprotein (A) And Oxidised Phospholipids Following Metabolic Surgery. (Poster Presentation at EAS Annual Meeting, Maastricht, 2019)

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15. Ho JH, **Adam S**, Azmi S, Dhage SS, Liu Y, Ferdousi M, Syed AA, Ammori BJi, Malik RA, Donn R, Soran H. The effects of bariatric surgery on obesity-related male sexual dysfunction. (Oral Presentation at British Obesity and Metabolic Surgery Society Annual Meeting, Telford, 2018)

16. **Adam S**, Soares J, Lake E, Del Col C, Yates A, Trainer P *et al.* The impact of a rapid cortisol assay on success rate, procedural efficiency and radiation dose in adrenal vein sampling for primary hyperaldosteronism. (Oral Presentation at British Society of Endocrine and Thyroid Surgeons Annual Meeting, Belfast, 2017)

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19. Soran H, **Adam S**, Durrington PN. A meta-analysis of the effect of intensive cholesterol reduction on numbers needed to treat to prevent cardiovascular events in 75439 participants in 10 randomised controlled clinical trials. (Featured Poster Presentation at EAS Annual Meeting, Prague, 2017)

20. **Adam S**, Minder A, Cottrell E, Davies A, Meredith S, Preziosi R *et al.* Salivary cortisone is a potential surrogate for serum cortisol measurement (Poster Presentation at SfE BES, Brighton, 2016).

21. Siahmansur TJ, Liu Y, Azmi S, Ferdousi M, Pemberton P, **Adam S** *et al.* Improvement in small fibre neuropathy and inflammatory biomarkers after bariatric surgery. (Oral Presentation at HEARTUK Annual Conference, Edinburgh, 2016)

Chapter 1: Introduction

1.1 Obesity: definitions and prevalence

The World Health Organisation (WHO) currently uses measurements of an individual's body mass index to define both overweight and obesity with measurements exceeding 25 kg/m^2 and 30 kg/m^2 respectively as diagnostic thresholds (1). The findings of the Global Burden of Disease (GBD) 2015 Obesity collaborators showed a marked increase in the prevalence of obesity between 1980 to 2015 across all sociodemographic index groups. Furthermore, using the GBD 2015 Obesity collaborators' data, approximately 1 in 10 persons worldwide were found to be obese (2, 3). Interestingly, Kelly *et al.* previously reported that the total number of obese adults in 2005 was 396 million and had forecasted that by 2030, 573 million adults would be obese (3). The GBD Obesity collaborators found that the number of obese adults in 2015 exceeded 600 million and this is similar to the WHO factsheet report using data from 2016 (1, 3). Therefore, it is clear that the prevalence of obesity has risen and is therefore likely to continue to rise.

1.2 Obesity: Associated Health Burden

Although the debate as to whether to recognise obesity as a disease rather than a risk factor continues, organisations such as the American Medical Association and the World Obesity Federation have already chosen to do so (classify obesity as a disease) (4, 5). Regardless of whether obesity itself is considered a disease or not, there is unequivocal evidence that obesity is associated with serious metabolic consequences including Type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), hypertension and dyslipidaemia (6). In addition to these commonly reported associations, there have been different relationships between obesity and other systemic and organ-specific conditions (Figure 1.1).

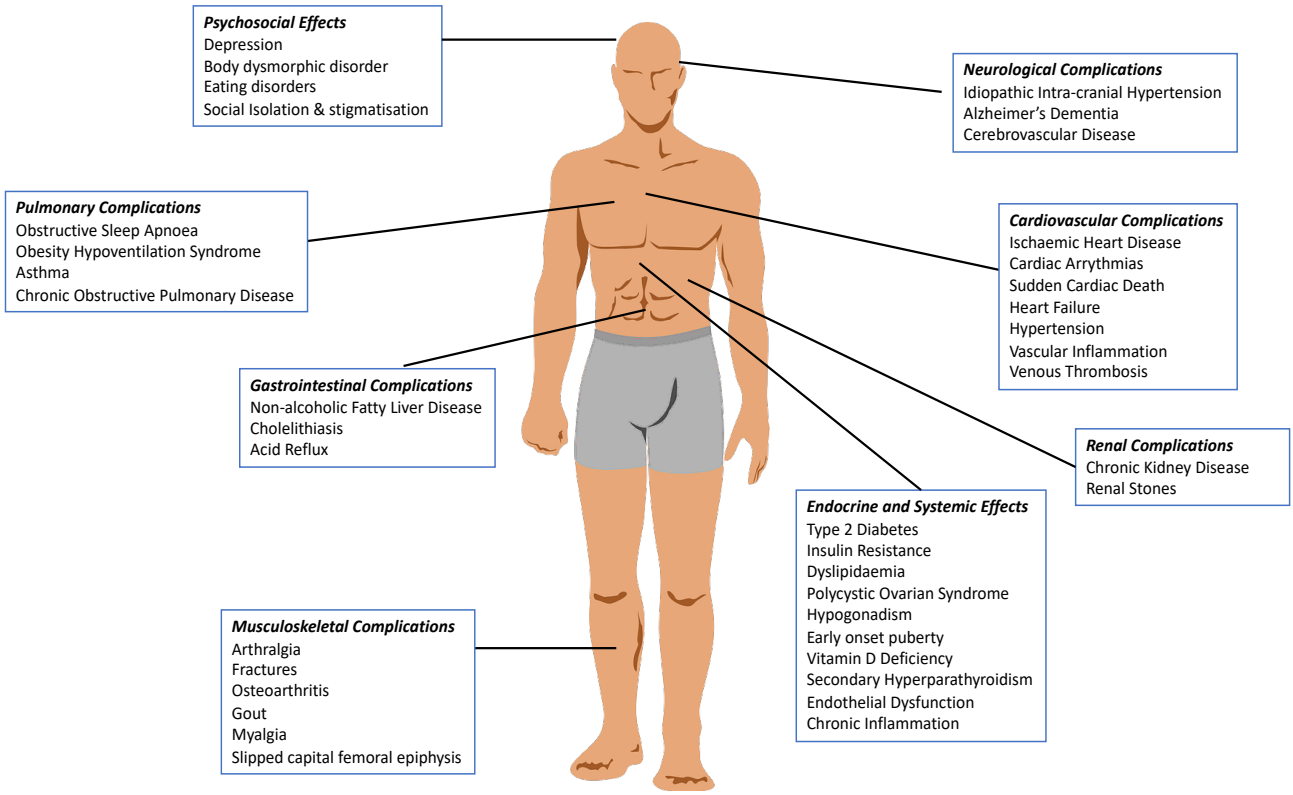

Figure 1.1: Obesity has association with multi-system complications with potentially serious health consequences.

Data derived from review of information in different articles (4, 6-26).

In addition, obesity is now an established risk factor for cancers of different organ systems, including renal, thyroid, multiple myeloma, meningioma, oesophageal, lower gastrointestinal, gastric, oesophageal, endometrial, ovarian, breast, biliary, renal, pancreatic and hepatic (27, 28).

1.3 Metabolic Complications of Obesity

Amongst the milieu of complications associated with the obese state, metabolic dysfunction is the principal one. The main metabolic complications are insulin resistance, T2DM and related microvascular disease, hypertension, and CVD.

For the purposes of this thesis, the metabolic complications of interest are obesity related CVD risk factors (especially lipoproteins and lipoprotein functionality), insulin resistance and the metabolic syndrome, T2DM and

related microvascular complications including and metabolic bone health factors, particularly vitamin D deficiency.

The metabolic syndrome is an umbrella term for a group of conditions that provide significant predisposition for atherosclerotic CVD. The National Cholesterol Education Programme Adult Treatment Panel III defines the metabolic syndrome as having 3 out of 5 risk factors (29) including central obesity, raised triglycerides, low high-density lipoprotein cholesterol (HDL-C), hypertension and dysglycaemia (Table 1.1). Interestingly, low-density lipoprotein cholesterol (LDL-C) is not included in the diagnostic criteria of the metabolic syndrome despite having a causal relationship to CVD (30). Furthermore, there remains a controversy about exactly whether mild-to-moderate hypertriglyceridaemia (which is usual in the metabolic syndrome) directly exerts an increase in CVD risk (31). In addition, HDL-C and the link with CVD protection is by association from large population studies however this relationship is now being re-examined (32). Levels of small-dense low-density lipoprotein (sdLDL) are enhanced when triglycerides are raised (33-35); these particles are particularly prone to atherogenic modifications (36, 37) and might provide some explanation as to increases in CVD risk in the metabolic syndrome.

Table 1.1: Components of the Metabolic Syndrome using National Cholesterol Education Programme Adult Treatment Panel III Criteria

Risk Factor	Reference Point	Additional Considerations
The diagnosis of metabolic syndrome requires at least 3 risk factors (out of 5).		
Waist Circumference	≥ 102 cm in men ≥ 88 cm in women	➢ Marker of central obesity. ➢ For Asian ethnicity, use cut-off point of ≥ 90 cm for men and ≥ 80 cm for women.
Hypertriglyceridaemia	≥ 1.7 mmol/l *or* using medication to reduce triglycerides	
Low HDL-C Levels	< 1.03 mmol/l in men *or* < 1.30 mmol/l in women *or* using medication for low HDL-C	➢ Note different criteria based on sex.
Raised Blood Pressure	≥130 mmHg systolic *or* ≥ 85 mmHg diastolic blood pressure *or* using an anti-hypertensive agent.	

Table 1.1 shows the key components of the metabolic syndrome. A diagnosis of the syndrome requires 3 out of 5 possible criteria (29). HDL-C: High-Density Lipoprotein Cholesterol; HbA1c: glycated haemoglobin. Adapted from (29).

The metabolic syndrome has now become well-established and this is largely due to the work of Gerald Reaven (38, 39). It is therefore important to understand the fundamental concepts that have led to our current understanding of the syndrome.

1.4 The Reaven Hypothesis: An Historical Perspective

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Author's Contribution: Safwaan Adam researched the available literature and co-wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript.

Handrean Soran, **Safwaan Adam**, Jan H Ho, Paul N Durrington

Commentary

The Reaven syndrome: An historical perspective

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SAGE

Handrean Soran[1,2], Safwaan Adam[1,2], Jan H Ho[1,2] and Paul N Durrington[1]

When reliable plasma insulin assays and accurate triglyceride methods became available in the 1960s, the scene was set for Gerald Reaven and colleagues to discover the association between the insulin response to carbohydrate feeding and serum triglyceride levels.[1] Higher insulin responses were associated with higher triglyceride levels. Initially, Reaven hypothesised that the increased insulin levels were the cause of the hypertriglyceridaemia, because insulin was believed at that time to stimulate hepatic very low density lipoprotein (VLDL) secretion.[1] However, in the 1980s, it became possible to culture adult hepatocytes without the necessity for insulin to maintain their viability and it was then evident that the primary effect of insulin on hepatic VLDL secretion was inhibitory.[2] This proved to be due to an increase in the proteolytic degradation of newly synthesised apolipoprotein B100 [the major protein moiety of VLDL and low-density lipoprotein (LDL)] before it could be assembled into VLDL.[3] Thus, hypertriglyceridaemia must be due not to hyperinsulinaemia, but to resistance to insulin. Reaven then argued that, like muscle, the liver must be resistant to the action of insulin, at least in regard to its diminished capacity to take up glucose. Increased insulin levels were thus a response to overcome this resistance in order to maintain euglycaemia [or the failed attempt to maintain normal glucose levels in the case of type 2 diabetes mellitus (T2DM)]. Hepatic insulin resistance releases the brake imposed by insulin on VLDL production and thus explains the hypertriglyceridaemia of metabolic syndrome and T2DM. This theory was later confirmed by human studies of VLDL kinetics.[3] Subsequently, a clinical syndrome emerged associated with an exaggerated insulin response to carbohydrate feeding. In addition to hypertriglyceridaemia, this syndrome comprised increased risk of atherosclerotic cardiovascular disease (CVD), T2DM or a predisposition to develop it, low high-density lipoprotein (HDL) cholesterol, non-alcoholic steatohepatitis, hypertension, hyperuricaemia, raised indices of inflammation and of coagulation (plasminogen activator inhibitor-1, fibrinogen), hirsutes and male pattern obesity in women [polycystic ovary syndrome, low sex hormone binding globulin (SHBG)] and in extreme cases acanthosis nigricans.[3,4] This, Reaven termed 'Syndrome X',[2] although it is now more widely known as the metabolic syndrome, particularly when associated with central obesity. That insulin resistance and the hyperinsulinaemia arising as a consequence are the causes of this syndrome is the current iteration of the Reaven hypothesis. Visceral adipose tissue is believed to release inflammatory cytokines, which, arriving at the liver in high concentration via the portal vein, oppose the anabolic actions of insulin.

Diabetologists will be familiar with the large doses of insulin required to make even modest improvements in hyperglycaemia in obese patients with T2DM, often far greater than are required in type 1 diabetes. However, while Reaven was developing his hypothesis, rarer syndromes involving insulin resistance came to light in which a hundred or more units of exogenous insulin may be required each day. Among these are insulin receptor mutations, which lead to hyperglycaemia, but not hypertriglyceridaemia, and abnormalities of body fat distribution, such as Dunnigan–Kobberling syndrome (due most commonly to mutation of LMNA which codes for lamins of the inner nuclear membrane) in which the insulin resistance is associated with both hyperglycaemia and hypertriglyceridaemia.[5] It thus became obvious that resistance to insulin-stimulated glucose uptake (and consequent increased insulin secretion) could arise at the pre-receptor level [e.g. by non-esterified fatty acid inhibition of glucose uptake (Randle effect)], at the level of the insulin receptor (due say to a gene variant) or occur as post-receptor phenomena within the cell where insulin signals to many processes by a variety of mechanisms. Indeed, when the insulin receptor is intact, some of these processes may be underactive due to insulin resistance to their particular signalling mechanism while others may be overstimulated because their regulatory pathway can still respond to the raised insulin levels. Furthermore, these

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1.5 Obesity, Bariatric Surgery and Emerging Cardiovascular Disease Risk Factors

1.5.1 Obesity and cardiovascular disease

We have learnt from large studies that obesity augments the risk of CVD (Figure 1.2) (40-42). Obesity provides a pathological environment in which a milieu of pathological factors (in relation to CVD) can exist (Figure 1.3) (43, 44). Furthermore, there is the possibility of a compound effect of risk factors such that an obese person with T2DM who is already prone to obesity-related dyslipidaemia has the additional detriment on lipoproteins provided by diabetes (45). This makes preventative measures challenging. An acknowledged limitation of a widely used CVD prediction tool, the QRISK2 calculator, is that it may underestimate CVD risk in those with a BMI >40 kg/m^2 (46).

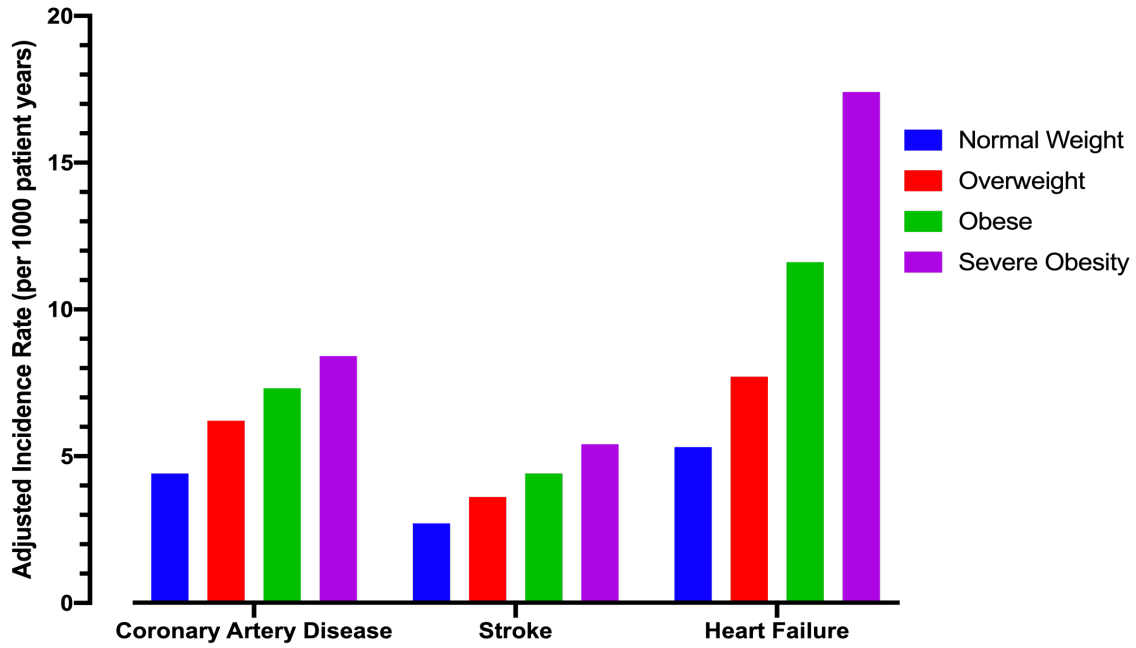


Figure 1.2. Incidence of cardiovascular disease sub-types according to BMI category.

Figure derived using data from Ndumele et al.(42) (under current copyright permissions).

In addition, in those with presenting acutely with an acute coronary syndrome (ACS), treatment of the vascular pathology provides its own distinctive problems. A previous study examining outcomes in a health registry of more than 200000 patients found that patients with a BMI >40 kg/m^2 were more likely to develop vascular complications (OR 1.89; p<0.0001), contrast-induced nephropathy (OR 1.89; p<0.0001), dialysis-requiring renal dysfunction (OR 4.08; p<0.0001) and greater mortality (OR 1.63; p<0.0001) (47). Additionally, there was almost double the percentage of patients with a BMI >40 kg/m^2 undergoing percutaneous coronary intervention in 2009 compared to 1998 and this has implications on the availability of adequate resources to cater for this unique cohort (for example, weight-limits on angiography tables) (47).

The key risk factors for CVD that are enhanced in obesity are highlighted below (Figure 1.3). The link between obesity and insulin resistance and dyslipidaemia respectively has been well-established (19, 43, 44) and these are already firmly embedded in CVD risk calculation algorithms (46). However, obesity also results in oxidative stress (48) as well as a state of chronic inflammation (49) which has become a target to prevent CVD. A recent randomised trial (n=10061) using the monoclonal antibody canakinumab showed that decreasing inflammation (defined by C-reactive protein [CRP] fall) using the drug, independent of lipid level changes, reduced the incidence of secondary CVD events by 7-15% depending on the dose used (50, 51).

Previous studies have introduced the concept of the “obesity paradox” in that those patients who were overweight were shown to have better outcomes after having suffered an ST elevation myocardial infarction (52). However, data from the United States National Cardiovascular Data Registry showed that patients with a BMI > 40 kg/m^2 had greater in-patient mortality figures (53). Furthermore, data from observational studies support an obesity paradox in relation to stroke (54) but the evidence is not robust in the case of severe obesity. In summary, there is no convincing evidence of the existence of an obesity paradox in severe obesity. Therefore, this notion is not applicable in a cohort of patients who would usually be eligible for bariatric surgery.

When considering the multiplicity of CVD risk conferred by obesity alone, any treatment that is focused on obesity (e.g. bariatric surgery) is then likely to result in a synergistic improvement in pathological factors which will lead to a net reduction in CVD.

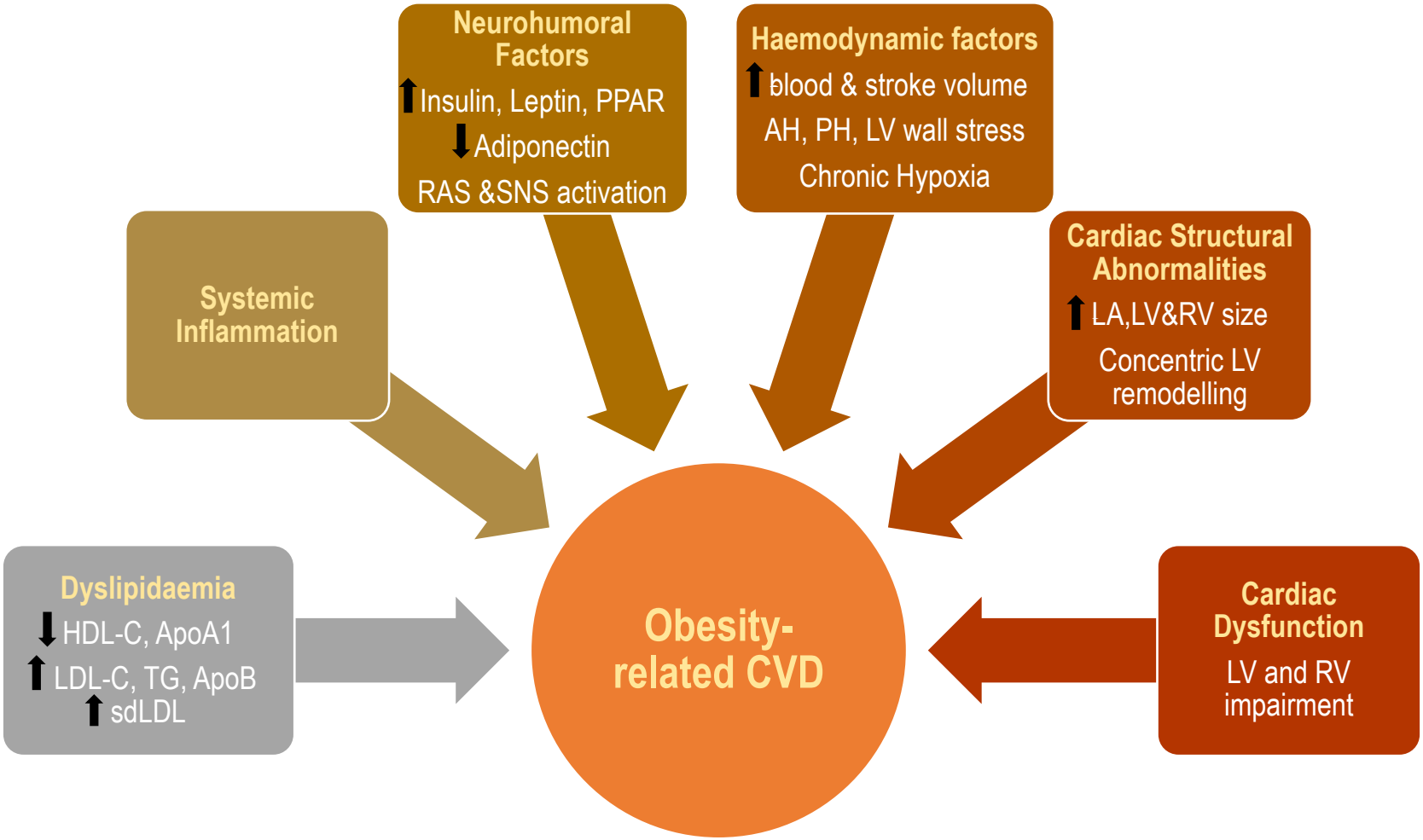


Figure 1.3. Known pathological factors that influence cardiovascular disease in obesity.

CVD incidence is increased in obesity via a number of pathological mechanisms. Information collated from topic reviewed in (43) and (44). HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; sdLDL: small dense low density lipoprotein; PPAR: peroxisome proliferator activator receptor; RAS: renin angiotensin aldosterone system; SNS: sympathetic nervous system; AH: arterial hypertension; PH: pulmonary hypertension; LA: left atrium; LV: left ventricle; RV: right ventricle.

1.5.2 The impact of bariatric surgery on cardiovascular disease

Bariatric surgery has been shown to have favourable effects on the incidence of subsequent CVD especially when compared to non-surgically managed controls (55). There are a number of improvements in CVD risk factors following bariatric surgery including body weight (55, 56), T2DM (57, 58), hypertension (56, 57, 59), dyslipidaemia (56-58) and reduction in atherogenic lipoproteins (60), endothelial dysfunction (60), obstructive sleep apnoea (56, 57), inflammation (49, 60, 61), levels of adiponectin (60) and proteinuria (62). The SOS study showed a clear reduction in both fatal (adjusted [for baseline variables] HR, 0.47; 95% CI, 0.29-0.76; *P* = .002) and total number of cardiovascular events (adjusted [for baseline variables] HR, 0.67; 95% CI, 0.54-0.83; *P* < .001) in patients who underwent bariatric surgery compared to matched controls (Figure 1.4).

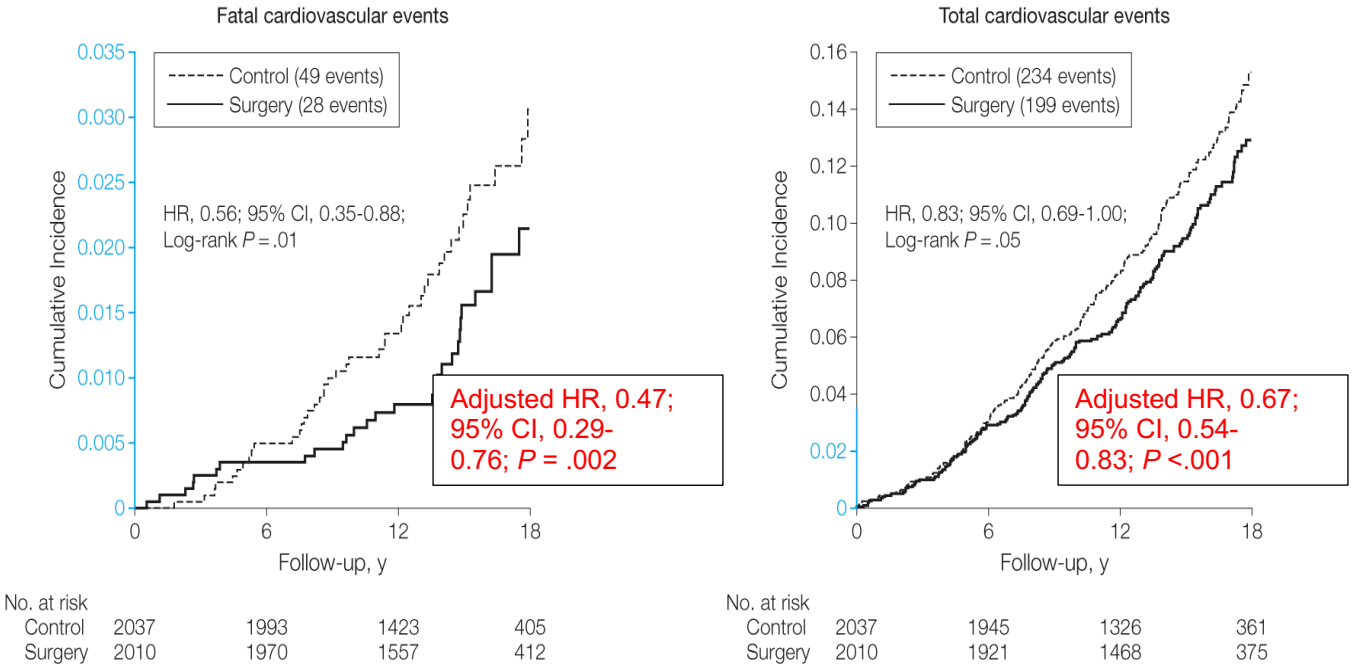


Figure 1. 4. Cardiovascular events after bariatric surgery.

Figure reproduced, with permission (see appendix), from Sjöström et al. (63). Cardiovascular events defined as combination of myocardial infarction and stroke.

Interestingly, a review of the key results from the SOS study revealed that the reductions in CVD following bariatric surgery were independent of weight loss with the exact mechanisms remaining unclear (55).

1.5.3 Emerging cardiovascular disease risk factors

1.5.3.1 High density lipoprotein functionality

Obesity is associated with low levels of HDL-C levels and apolipoprotein A1 (apoA1), which is the major protein component of HDL (64). The landmark Framingham Study showed an inverse relationship between HDL-C and CVD risk (65). Consequently, randomised trials have studied the effect of pharmacologically raising HDL-C levels with either niacin, niacin and laropripant combination or dalcetrapib but did not show CVD benefit (66-68). In the Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/High Triglycerides: Impact on Global Health Outcomes (AIM-HIGH) trial (n=3414), the addition of niacin to intensive statin treatment was compared to statin treatment alone (66). Despite a 16% increase in median HDL-C levels there was no reduction in the incidence of CVD and indeed the study was terminated prematurely due to lack of efficacy (66). Schwartz *et al.* used the cholesterol ester transfer protein (CETP) inhibitor dalcetrapib in a randomised control trial involving 15871 patients who had suffered a recent acute coronary event (68). Even though the treatment group had increases in HDL-C of between 31 and 40% (compared to 4-11% in placebo group), there was no reduction in incident CVD (68). The Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events (HPS2-THRIVE) study (n=25673) used a combination of niacin and laropripant as an adjunct to high-intensity statins in patients at high risk of CVD. The incidence of major vascular events (defined as fatal and non-fatal myocardial infarction/coronary disease, stroke or arterial revascularisation procedures) was compared to patients' treatment with high-intensity statin treatment alone. Similar to the AIM-HIGH study there was no treatment benefit despite an average increase of HDL-C by 0.16 mmol/l in the niacin/laropripant group (67). The Randomised Evaluation of the Effects of Anacetrapib through Lipid Modification (REVEAL) trial randomised 15225 patients to anacetrapib (and atorvastatin) and compared them to 15224 patients who were taking placebo alongside atorvastatin (69). The authors reported a relative increase of HDL-C of 104% and relative reduction in non-HDL-C of 18% in the anacetrapib group (69). Additionally, the anacetrapib group suffered fewer coronary events (10.8% vs 11.8%) than the placebo group. However, despite the marked increase in

HDL-C, the reduction in coronary events was felt to be more as result of a reduction in the non-HDL-C rather than the increase in the HDL-C (69, 70).

Accordingly, genetic predispositions to low HDL-C levels (due to deficiencies in apoA1, ATP binding cassette [ABC-] A1 and lecithin-cholesterol acyltransferase [LCAT]) do not reliably amplify CVD risk. (71). Moreover, SCARB1 gene mutations (which is associated with raised HDL-C levels) led to a higher frequency of CVD (72). Likewise, in a previous Mendelian randomisation study genetic tendency towards elevated HDL-C levels did not offer added protection against CVD (73). Therefore, there has recently been a greater emphasis on HDL functionality as opposed to HDL-C cargo alone.

HDL particles have a multitude of functions that are protective against CVD (74-78). These include protection against inflammation, thrombosis, oxidative stress alongside other benefits (Figure 1.5) (78).

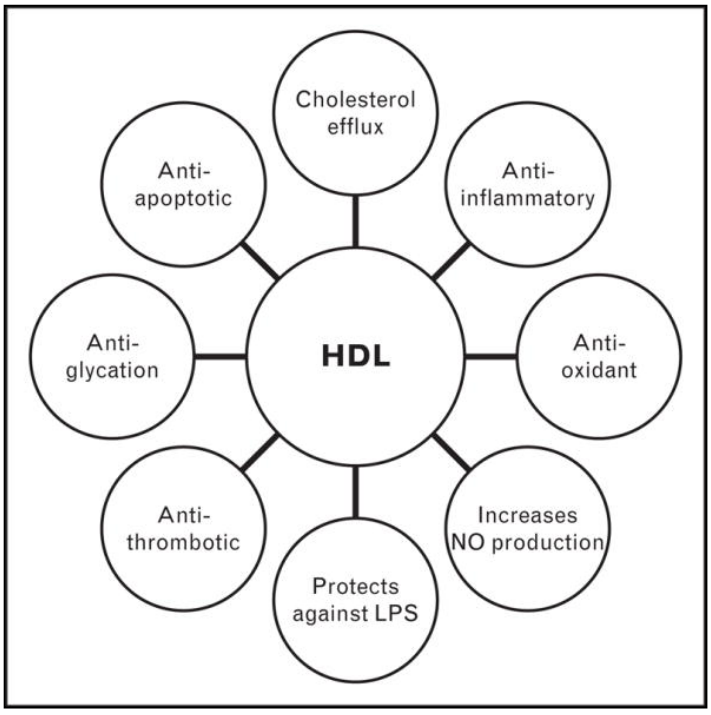


Figure 1. 5. Key functions of high-density lipoprotein.

Figure reproduced from Soran et al. (78) with permission (see appendix). HDL: high-density lipoprotein; LPS: lipopolysaccharide.

One of the key HDL functions is reverse cholesterol transport (RCT). This is the pathway through which surplus cholesterol is removed out of the circulation (78, 79). Cholesterol efflux capacity (CEC) is the key factor in RCT and symbolises the capacity of HDL to accept cholesterol from peripheral cells. RCT encompasses a process in which passive diffusion and active transport enabled mainly by ABCA1, ABCG1, scavenger receptor B1 (SR-B1) and CETP (Figure 1.6) (80).

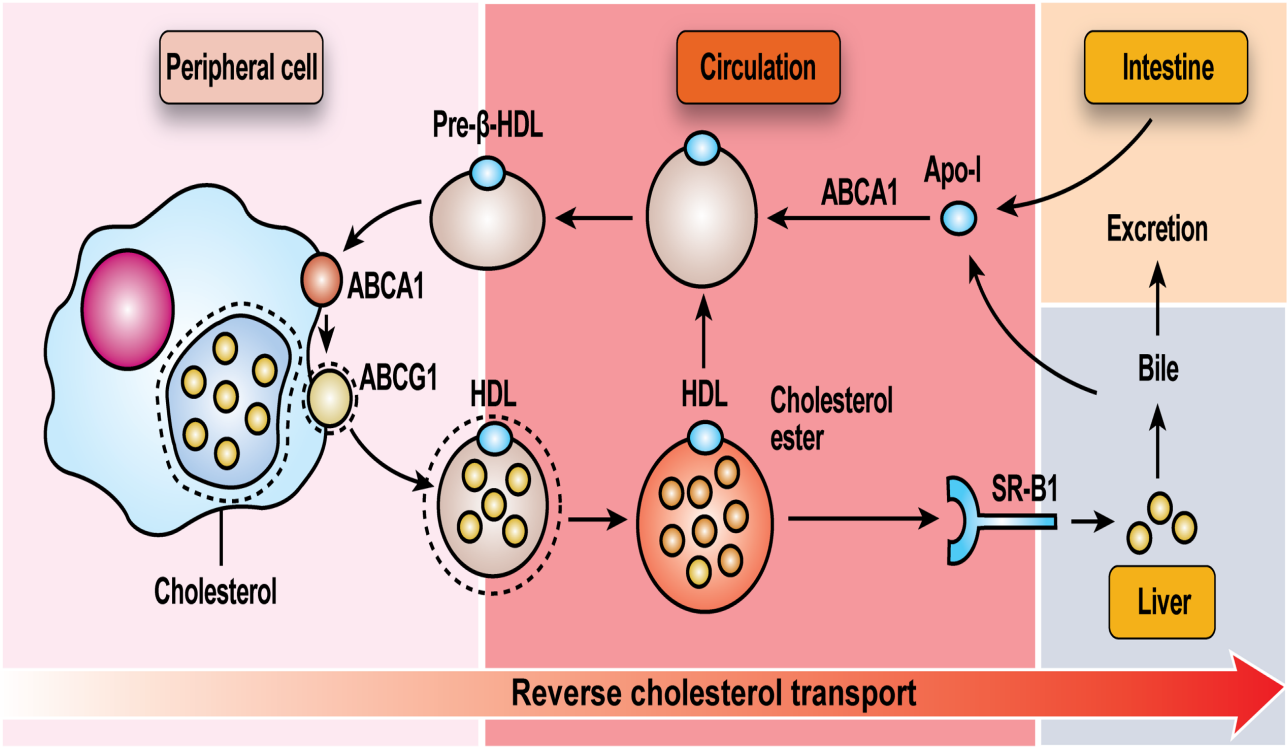


Figure 1.6.Key steps in the reverse cholesterol transport pathway.

Lipid-poor pre-β-HDL removes cholesterol from lipid rich macrophages in vessel walls and this process is mediated by ABCA1 and ABCG1. The resultant lipid-rich HDL particles are then removed from circulation via the liver through the actions of SR-B1. An alternate pathway for cholesterol to re-enter the circulation is mediated by CETP. Figure adapted, with permission, from Heinecke JW (80). HDL: high-density lipoprotein; Apo: apolipoprotein; ABCA1: ATP binding cassette receptor A1; ABCG1: ATP binding cassette receptor G1; SR-B1: scavenger receptor B1.

Importantly there are studies that have tested the hypothesis that impaired HDL functionality could predispose to CVD. In a study by Khera *et al.* (n=996), a reduction in CEC was related to coronary and carotid atherosclerosis (81). Furthermore, population-based studies (Dallas Heart and EPIC-Norfolk) have shown an

independent association between CEC and incident CVD (82, 83). Moreover, paraoxonase-1 (PON1) is an enzyme which is present in HDL with an antioxidant function and reductions in its activity have been associated with CVD (84).

1.5.3.2 The role of anti-apoA-1 IgG antibodies in cardiovascular disease

Autoantibodies against apoA-1 (anti-apoA-1 IgG) have been shown to be independent risk factors for CVD and mortality (85-87). Anti-apoA-1 IgG antibodies were initially related to atherosclerosis and CVD in people with autoimmune disease (88, 89). These antibodies were then shown to have a link with CVD and mortality in population-based studies (out of solely an autoimmune disease context) (86, 87). In a study of 221 patients with myocardial infarction, the presence of anti-apoA-1 antibodies was associated with a higher incidence of subsequent cardiovascular events within the first 12 months (85). Likewise, in another study, patients with acute coronary syndromes were shown to have higher levels of anti-apoA-1 IgG antibody levels (90).

A previous study in patients with recent myocardial infarction showed that in those patients who displayed anti-apoA-1 IgG positivity had high-risk inflammatory markers; anti-apoA-1 IgG antibodies were found to promote inflammation via interaction with immune receptor complexes (TLR2/CD14) (91). Furthermore, Batuca *et al.* showed that in patients with systemic lupus erythematosus (n=55), the levels of anti-apoA-1 IgG antibodies were inversely correlated with serum PON activity (92). Moreover, anti-apoA-1 IgG antibodies have been shown to modulate atherothrombosis and have a role in plaque instability (93, 94). Importantly, the anti-apoA-1 IgG antibodies have been related to oxidised LDL which has a role in atherosclerosis (95). In obesity, anti-apoA-1 IgG antibodies levels were higher, and their presence was associated with a coronary artery calcification (measured by calcium scoring) (96).

1.6 Obesity and Type 2 Diabetes

The risk of developing T2DM is increased 93-fold in women and 42-fold in men who are severely obese compared to healthy weight individuals (97, 98). It is still unproven what the exact mechanisms are behind the increase in prevalence of T2DM in obese persons (compared to normal-weight individuals) (99). The different proposed pathways that lead to T2DM in obesity have included the pathological effects of systemic inflammation, changes in the distribution and functionality of adipose tissue, disturbance of mitochondrial function and increases in free fatty acids (as is seen in obesity) inducing insulin resistance (99-101) (Figure 1.7). It is also unclear what the trigger for the onset for T2DM is in obese persons.

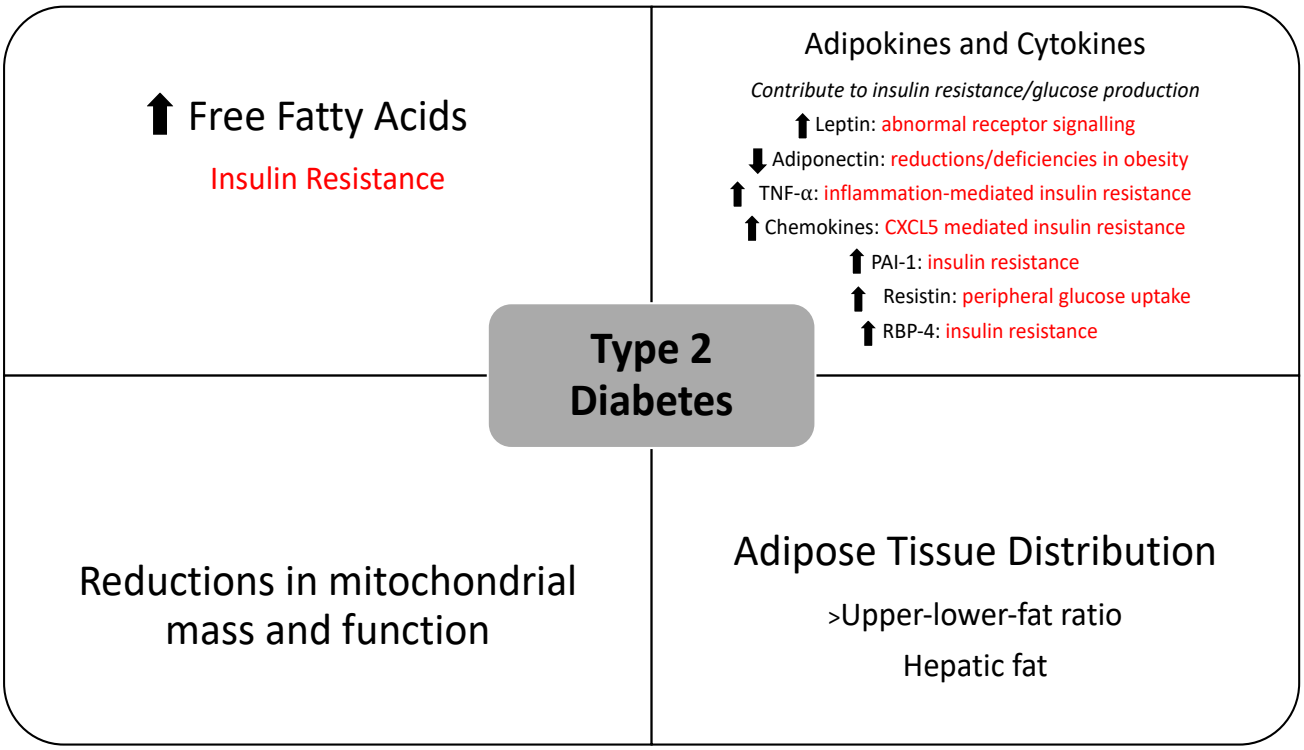


Figure 1.7. Potential pathways influencing pathogenesis of T2DM in obesity

The figure shows the possible factors that explain the close link between obesity and Type 2 Diabetes Mellitus (99-101). TNF-α: tumour necrosis factor alpha; PAI-1: plasminogen activator inhibitor-1; CXCL5: CXC ligand 5; RBP-4: retinol-binding protein 4.

1.6.1 Does weight loss improve Type 2 Diabetes?

Weight loss, whether surgically induced or due to caloric restriction, has proven to be effective at improving hyperglycaemia (102). Recently the Diabetes Remission Clinical Trial (DiRECT) study has shown impressive diabetes remission rates with intensive lifestyle modification by calorie restriction (103, 104). In this study, 149 participants were initially restricted to between 825 and 853 kcal/day for 12-20 weeks followed by structured food re-introduction and weight maintenance phases. Twenty-four months following the intervention, 36% of participants were in remission from T2DM (104). This supports other studies which also show a beneficial effect of calorie restriction on T2DM (105, 106).

It is more than 20 years ago that Pories *et al.* showed the marked improvement in insulin resistance promptly induced by bariatric surgery (107). The three major bariatric procedures have been illustrated below (Figure 1.8).

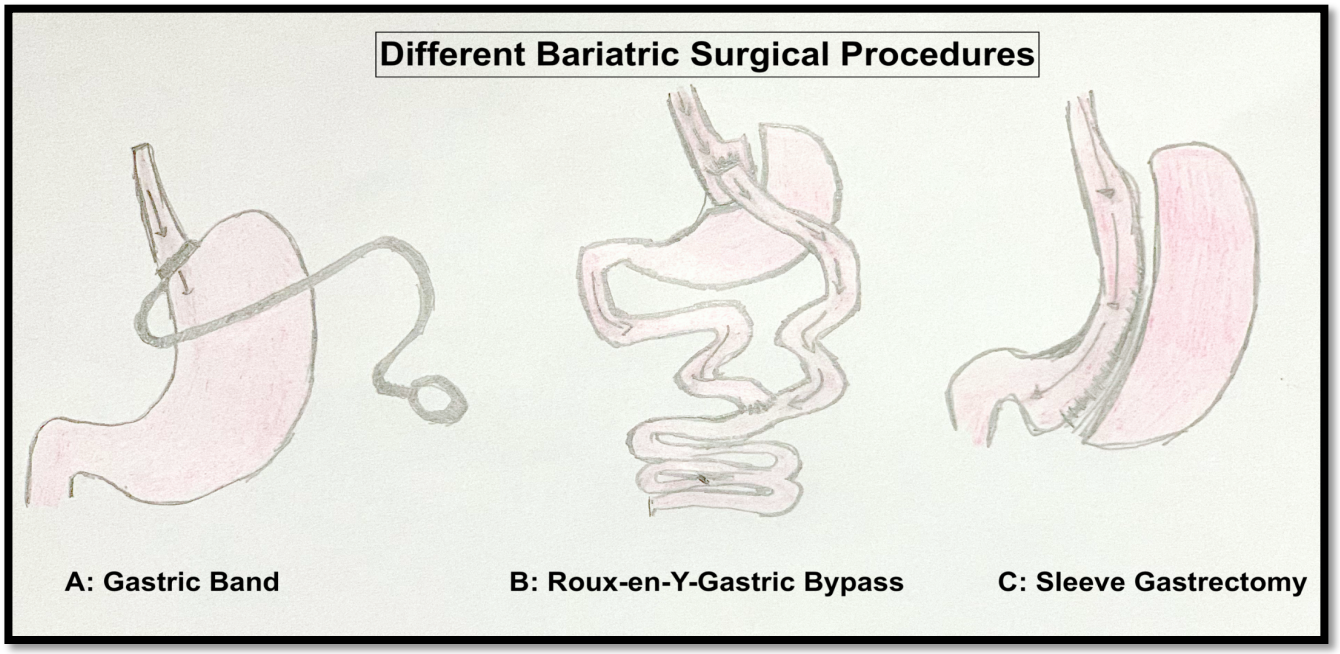


Figure 1.8. Bariatric Surgical Procedures

Figure 1.8 illustrates the three common bariatric procedures. Gastric band and sleeve gastrectomy are primarily restrictive procedures whilst Roux-en-Y Gastric Bypass is both malabsorptive and restrictive.

Since then there have been a number of short-term and longer-term studies in different settings that have shown unequivocal proof that bariatric surgery can cause improvement and remission of T2DM (55-58, 60, 108-118). A previous meta-analysis has shown remission from type 2 diabetes (T2DM) to be 92% (in 8 randomised studies; n=206) and 86% (in 43 observational studies; n=9037) (119). Indeed, it is apparent that the improvements in T2DM occur within the immediate weeks and months (Figure 1.9) following bariatric surgery (57, 107).

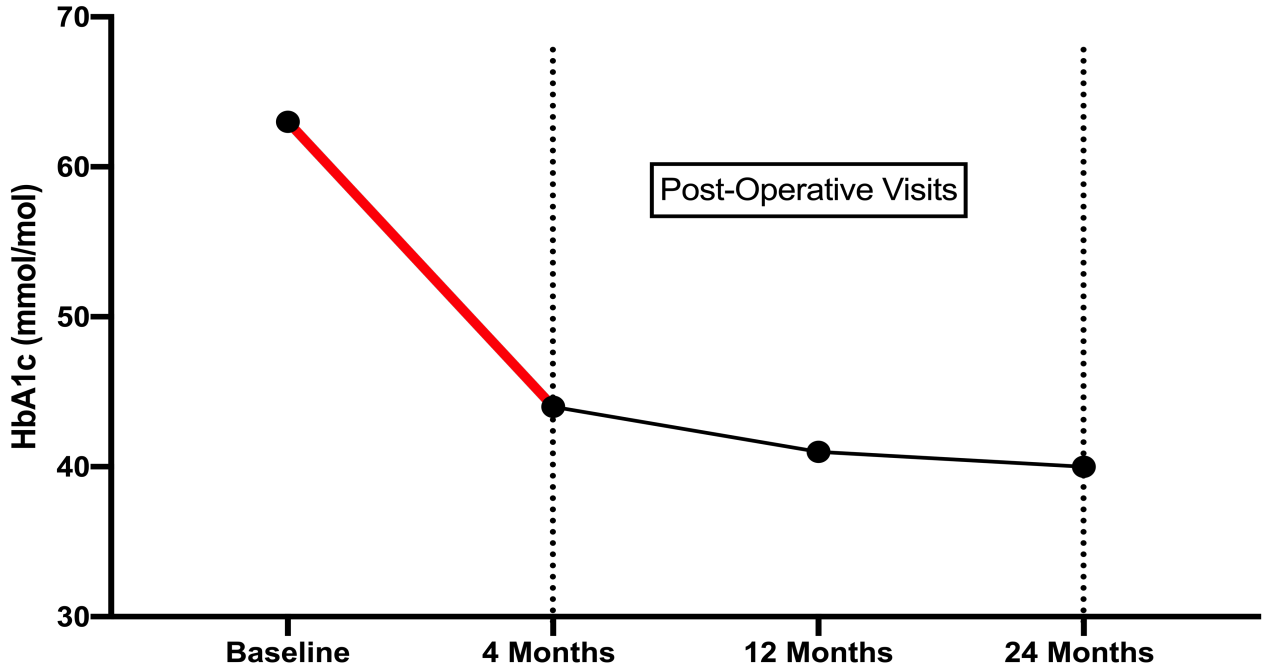


Figure 1.9. Improvements in glycated haemoglobin following bariatric surgery.

Data for image extrapolated from Kennedy-Dalby, Adam et al. (61). The thick red line denotes sharp gradient of fall in HbA1c in the first 4 months following bariatric surgery. Following this initial decline in HbA1c, there is relative plateauing of the HbA1c.

Given the relatively rapid improvement in glycated haemoglobin (HbA1c) following bariatric surgery, it is possible that weight-independent mechanisms may play a key role. It is unclear what these mechanisms may be and consequently there have been a number of hypotheses as to what the principal factors are (120).

1.6.2 Possible mechanisms for improvement in T2DM following bariatric surgery

The means by which bariatric surgery induces improvement in T2DM is still not fully understood. It is likely that there is a complex interplay between different factors that occur concurrently with a resultant benefit. There have been different theories (Figure 1.10) as to what the possible reasons may be. It is difficult to overlook the effect of calorie restriction alone, especially with the recent evidence from the DiRECT study (103, 104). Intriguingly, even prior to the results of the DiRECT study, some had suggested that caloric restriction and weight loss provided the main explanation for post-operative T2DM remission (121, 122). Additionally, given the changes in anatomy it is essential to consider the effect of observed changes in physiology and microbiology on T2DM.

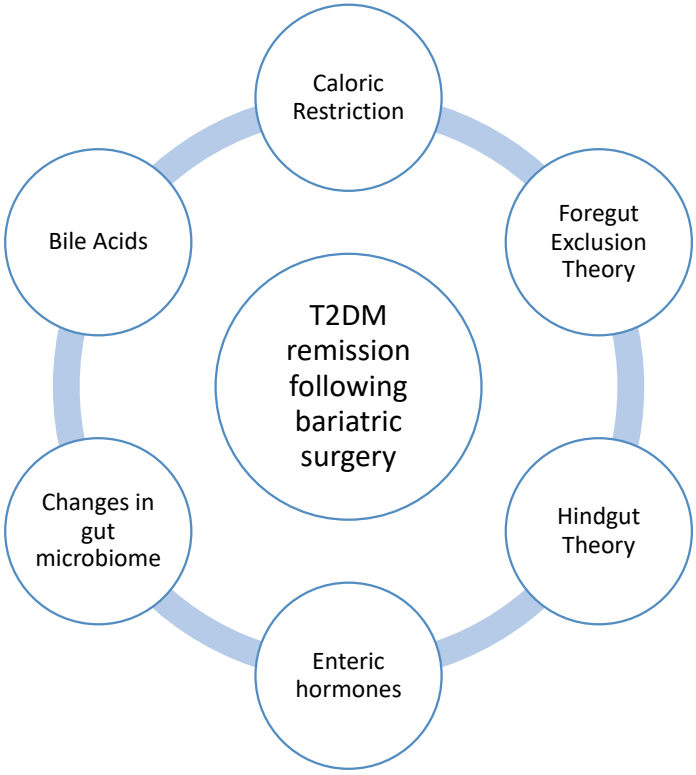


Figure 1.10. The different mechanisms by which there is improvement in and remission from Type 2 Diabetes following bariatric surgery.

1.6.2.1 Role of the Gut Microbiota

The study of gastrointestinal microbial changes in disease states is an exciting facet of research. The human gastrointestinal tract is colonised by a multitude of bacteria which on the whole serve to protect against pathogenic organisms and maintain homeostasis by promoting immunity and defending against systemic infection (123). From metagenomic studies we have learnt that the vast majority of the gut microbiota belong to two distinct bacterial families, Bacteroidetes and Firmicutes (124-126). Disturbance of these bacteria has been associated with the development of inflammatory bowel disease, arthritis and allergic tendency amongst other conditions (123). Importantly, there has also been an association with the development of obesity (127, 128), T2DM (129) and non-alcoholic fatty liver disease (130) which are all inter-related. For T2DM specifically, it has been postulated that reduced diversity of gut microbiota confers a risk to chronic low-grade inflammation which in turn augments the risk of T2DM (126, 131). After RYGB, the richness of the gut microbiome is augmented (132) which is relevant as reduced richness of the gut microbiome has been linked to metabolic complications of obesity (including T2DM) (133).

Changes in the gut microbiota have been shown within a day of dietary modification in humans (134). In mice, following bariatric surgery, rapid changes in the intestinal microbiota were shown at 1 week (135). Although the evidence at current is mainly based on association only, modulation of the gut microbiome remains a possible mechanism by which improvement in T2DM (particularly rapid improvement) occurs.

1.6.2.2 The role of bile acids

In a previous study by Gerhard *et al.* patients with T2DM showed reduced levels of circulating bile acids and fibroblast growth factor 19 (136). Bariatric surgery has been shown to increase circulating bile acid levels (137). Indeed, in a recent randomised trial, Nemati *et al.* showed a link between improvements in bile acids following bariatric surgery and reductions in HbA1c (138). This supports the findings of Gerhard and colleagues who showed that those who remitted from T2DM following bariatric surgery had higher levels of circulating bile acids than those who did not remit (136). Furthermore, Farnesoid X Receptor (FXR), a bile acid receptor, has been shown to be a mediator of improvements in T2DM following sleeve gastrectomy (139, 140).

1.6.2.3 The Foregut Exclusion Hypothesis

This theory indicates that the nutrient bypass of the duodenum and proximal jejunum is the reason for reduction in insulin resistance post bariatric surgery. Goto-Kakizaki rats, Rubino *et al.* (141) showed a marked difference in glycaemic control depending on the surgical procedure undertaken. Rats that had duodeno-jejunal bypass (DJB) performed (using a simple gastro-jejunostomy with duodenal exclusion) had superior glucose tolerance compared to rats who had either a simple gastro-jejunostomy without duodenal exclusion (GJ) or a 'sham' operation in which the normal passage of food was allowed despite surgery. Importantly, when the animals that had DJB switched to a GJ, there was deterioration in glycaemic control with a concomitant improvement in the rats that had the GJ transformed to a DJB. The results were independent of calorie restriction or weight loss. This experiment, therefore, infers

that there may be an unknown diabetogenic stimulus within the proximal small bowel.

Despite all of the different types of bariatric surgery having beneficial effects on glucose metabolism, Roux-en-Y gastric bypass (RYGB) and bilio-pancreatic diversion (BPD) have been shown to be the most effective (142-145). The DJB in rats mimics the RYGB and BPD in humans, hence, in spite of there being no similar studies in humans, this theory is believed to be the reason behind the difference in the improvement of insulin sensitivity.

1.6.2.4 The Hindgut Theory

Following on from the observation that RYGB and BPD provide superior glycaemic control compared to other types of surgery, one proposal is that this is a consequence of the expedited delivery of ingested nutrients to the distal ileum. Two rat experimental studies (146, 147) have implemented ileal transposition (IT) to test this theory. Both studies showed an improvement in insulin sensitivity following IT. Another study in which laparoscopic IT was performed in 60 obese men and women, demonstrated marked improvements in T2DM with a reduction in insulin resistance. It also exhibited substantial rises in glucagon like peptide 1 (GLP-1), glucose-dependant insulinotropic peptide (GIP) and peptide YY (PYY) which are produced by the L-cells in the distal ileum study (148).

Interestingly, Rubino and colleagues. in their study to prove the foregut exclusion theory (141) dismissed the hindgut theory on the basis that if rapid delivery of ingested food to the terminal ileum was the only reason for better glycaemic control, there should not have been any difference observed in the procedures used in their

study as both types of procedure (DJB and GJ) would achieve this objective (i.e. rapid delivery of food to the ileum).

1.6.2.5 Enterohormones

Another proposition is that the enterohormones have the predominant role in elucidating the disparity between diet and surgery in the matter of insulin sensitivity. Several hormones are relevant to appetite, energy and glucose homeostasis.

- The Incretins (GLP-1 and GIP)

These hormones are fundamental in terms of glucose control. The incretin effect is one in which insulin secretion is increased and glucagon secretion is suppressed. The end product is lower blood glucose. There is a reduced incretin effect in type 2 diabetes (149) and this has been a well-established area of targeted therapy for the condition. In addition to the studies mentioned above, other studies have suggested that gastric bypass surgery results in increased levels of GLP-1 (150-152) and GIP (152, 153).

- Peptide YY

This peptide promotes satiety and slows gastric emptying. It is produced in the terminal ileum and has been implicated in the sustained weight loss seen with bariatric surgery compared to diet treatment alone (154). This, in turn, helps with long term glucose homeostasis. A study in mice showed that there may be a role for PYY3-36, independent of its anorexic action, in enhancing the effect of insulin on glucose. PYY levels have been shown to increase after RYGB (155, 156).

- Ghrelin

Ghrelin has been shown to increase the hunger stimulus (157). Ghrelin levels rise before and fall after a meal and has therefore been associated with the urge to begin

eating (158). Ghrelin has also been shown to reduce insulin levels and raise plasma glucose (159). Patients with diet induced weight loss have higher levels of circulating ghrelin compared to those who undergo bariatric surgery (160, 161). This is especially important in the context of glucose metabolism.

Whether all of these hormonal changes are only due to anatomical re-structuring is unknown but it has been proposed that the variations may be due to gut adaptation because there has been a previous description of intestinal cell hyperplasia post gastric bypass surgery (162). The role of stricter caloric restriction post gastric bypass compared to those on diet treatment only has also been implicated as a potential factor in increasing the levels of the incretin hormones (152).

1.7 Obesity, Microvascular Complications of Type 2 Diabetes and Bariatric Surgery

It is difficult to fully quantify the independent effect of obesity on the microvascular complications of T2DM as the majority of patients with T2DM are obese (97, 98). Furthermore, in obesity, there are a constellation of other metabolic factors that also possibly exert their own risk of microvascular disease. Glycaemic parameter targets are borne out of prevention of microvascular disease (163); it is imperative therefore that any intervention carried out for T2DM must show benefit to microvascular disease. In obese people, weight-loss (especially by bariatric surgery) allows for the concurrent correction of metabolic risk factors (56, 58) thus potentially allowing for favourable effects on established microvascular disease and protection against incident disease.

1.7.1 Retinopathy

Diabetic retinopathy (DR) affects more than a third of people with diabetes and is the leading cause of sight loss in people of working age (164, 165). The most significant risk factors for the development of and progression of DR are the duration of diabetes, glycaemic management and blood pressure control (165). Long-term outcome analysis of patients with type 1 diabetes (T1DM) in the Diabetes Control and Complications Trial (n=1441, of which 726 did not have DR vs 715 who had mild DR) showed that reduction in HbA1c both deferred the onset of as well as reduced the progression of DR (163). These conclusions are substantiated by the United Kingdom Prospective Diabetes Study (UKPDS retinopathy analysis; n=3709 of which 2316 did not have retinopathy at baseline) which showed that a 1% (11mmol/mol) reduction in HbA1c equated to a 31% reduction in DR (166). UKPDS also emphasised the importance of total glycaemic exposure indicating that early intervention (with subsequent reduction in duration of glucose exposure) reduces the development and progression of DR (166). Similarly, in UKPDS, reductions in systolic blood pressure of 10mmHg equated to a reduction in photocoagulation treatment or vitreous haemorrhage (both markers of increasing severity of DR) by 11% (166).

The evidence regarding obesity as a risk factor for DR is inconsistent. A recent meta-analysis by Zhu *et al.* (13 prospective cohort studies, 14575 participants) reported a 20% relative increase in the incidence of DR due to obesity (167). However, on sub-analysis, there was no significant risk conferred by obesity on proliferative DR (167). Interestingly a previous study in Singapore (n=420) in patients with T2DM, a raised BMI conferred protection against DR (168). In that study however, in women, a higher waist-to-hip ratio was associated with DR (168). A study (n=592) in patients with Type 1 diabetes mellitus (T1DM) showed that being overweight increased the risk of DR (169).

Bariatric surgery is primarily indicated in clinical guidelines as a treatment for obesity (170) however given the impressive results with T2DM, it can also be viewed as a primary treatment for T2DM (107, 120). Results for its effect on DR are differing. Conceptually, a rapid improvement in HbA1c can lead to worsening of DR especially considering the risk of hypoglycaemia after surgical weight-loss. There is one randomised controlled trial with a two year follow-up period that showed that bariatric surgery did not significantly change DR outcomes in that time compared to medical management of diabetes and secondly that for the vast majority of patients (87%) their pre- and post-operative retinal status remained static (110, 171). Observational data include a short pilot study in 2012 which showed that compared to the pre-operative findings, within 12 months (assessments done from 6-12 months) of surgery, 2 out of 15 patients had new-onset DR whilst 2 out of 7 patients with DR showed evidence of progression (172). More recent short-term (1-3 years) observational follow-up studies have shown that following bariatric surgery there is little progression of DR (173-176) with one of these showing that surgical management was favourable to medical management of diabetes in the context of DR (173) but two others showing no significant differences (177, 178). There is a dearth of evidence with regards to long-term outcomes of DR following bariatric surgery. In one study which compared laparoscopic adjustable gastric band (LAGB) to medical management, similar outcomes were found between both groups with only one out of 87 participants in the medical group developing new DR compared to none in the surgical group. Despite the impressive time period analysed, the key limitation when assessing the results of this study is that currently LAGB is much less popular as weight loss surgery compared to RYGB and laparoscopic sleeve

gastrectomy (LSG) due to superior efficacy found in the latter two (179). A prospective observational study, albeit with a small number of patients, showed that at both 3 (n=26) and 5 years (n=13) of follow-up there was no progression of DR. In addition, though not exclusive for DR, the Swedish Obese Subjects (SOS) study (mean follow-up 17.6 years) showed a significant reduction in all microvascular complications of diabetes in those who had bariatric surgery (n=288) compared to a group who did not have surgery (n=55). Merlotti *et al.*, in a meta-analysis of seven controlled studies (n=2966 participants) found that bariatric surgery prevented new cases of diabetic retinopathy but had no effect on either progression or regression of retinopathy (180). The meta-analysis was limited by a relatively short duration of follow-up in the in the studies with only one study having a follow-up period of more than 5 years (180), therefore limiting the interpretation of the results due to insufficient longitudinal follow-up. Furthermore, there was little emphasis in the included studies on sight-threatening DR (only one study assessed for this in any detail) which is arguably the main outcome of interest in such an analysis.

There is still a need for a well-designed and sufficiently powered longitudinal study to comprehensively gauge whether indeed bariatric surgery does confer benefit upon DR.

1.7.2 Diabetic Kidney Disease

There is much morbidity attributable to diabetic kidney disease (DKD). Additionally, decline in kidney function (of any cause) has been associated with all-cause mortality (181, 182) and this has also been observed specifically in DKD (183). DKD increases the risk of CVD with proteinuria, a key feature of DKD, having been established as a CVD risk factor (181-183).

A strong relationship between obesity and nephropathy was described more than thirty years ago (184). Obesity causes a separate disease entity called obesity related glomerulopathy of which the histological hallmark is focal segmental glomerulosclerosis (185, 186). Data from the United Kingdom National Diabetes Audit examining patients with both T1DM (n=58791) and T2DM (n=733769) showed

an increase in prevalence of DKD in obese persons with either type of diabetes (187). Notably, in patients with T2DM, obesity was associated with an increased prevalence of all stages of chronic kidney disease (CKD) (187). For T2DM patients with a normal estimated glomerular filtration rate (eGFR), there was a 10% relative increase in the prevalence of microalbuminuria in obese compared to non-obese persons; in patients with end-stage renal disease (stage 5 CKD), there was an 88% relative increase in prevalence (187).

Bariatric surgery has proven to be very effective for proteinuria in the context of DKD (62). In the meta-analysis by Upala *et al.* there were 15 included studies (3 with control arm included; total n = 1839) with a mean study follow-up duration of approximately 40 months. The longest follow-up duration was 10 years (3 studies) and 9 of the studies had a follow-up period of 24 months or less (thus limiting durability of surgery interpretation). This study showed a mean reduction in the urinary-albumin-to-creatinine ratio (uACR) of approximately 7 mg/g (62). Another retrospective study (n=163; mean follow up of 3 years) showed that there were improvements in both glomerular hypo- and hyperfiltration after bariatric surgery (188).

An essential consideration is that estimations of the GFR have limitations in obese patients as creatinine-based methods alone have not been validated in this cohort. Furthermore, following bariatric surgery there is likely to be loss of muscle mass and as such the reductions in creatinine may not entirely reflect renal function. Friedman *et al.* compared different eGFR measurements against measured GFR (plasma iohexol clearance) in a study of 36 subjects before and after bariatric surgery (189). The eGFR measurements were either creatinine based, cystatin C based or used a combination of creatinine and cystatin C. The authors found that Chronic Kidney Disease Epidemiology Collaboration 2012 equation combining both creatinine and cystatin C (CKD-EPI$_{cyst\text{-}creat}$) was the best predictor of mGFR both before and after bariatric surgery (189).

1.7.3 Diabetic Neuropathy

Diabetic neuropathy (DN) is the most common form of neuropathy in the developed world accounting for more hospital admissions than any other microvascular complication of diabetes (190, 191). It results in the largest number of non-trauma related amputations (190) and causes much morbidity for patients including mood disorders (192, 193), poor quality of life (193, 194) and physical disability (190, 195). There is also a higher rate of mortality in sufferers of DN compared to those without it (196).

DN can broadly be classified as follows (190):

1. Distal symmetrical DN
    a. Large Fibre
    b. Small Fibre
2. Proximal motor DN
3. Entrapment neuropathies
4. Mononeuropathies

The commonest variant is the distal symmetrical DN with subclassification based on which type of nerve fibre is affected (large versus small nerve fibre) (190). Usually patients have a combination of both small and large fibre pathology and therefore present with a mix of clinical symptoms and signs (190). The differences between the two subtypes of distal symmetrical DN are summarised below (Table 1.2).

Table 1.2. Differences between small and large nerve fibre pathology.

	Large Fibre	Small Fibre
Type of Fibre	Large, myelinated Aα/ß Sensory or Motor	Small, sparsely myelinated Aϑ/C Sensory and autonomic
Symptoms	Weakness of muscles of hands and feet "walking on cotton wool" Difficulty in tiptoeing	Early: Superficial pain and burning, increased pain sensation. Late: numbness, reduced pain sensation
Signs	Sensory ataxia Impaired vibration perception Impaired proprioception Muscle wasting of small muscles of hands and feet Feet feel warm to touch	Impaired cold and warm sensation Autonomic findings: reduced moisture of skin, impairment in deep-breathing heart rate variability, orthostatic hypotension Deep tendon reflexes preserved Feet feel cold to touch
Nerve Conduction Studies	Nerve conduction velocities impaired	Nerve conduction velocity preserved
Special Considerations	Higher risk of Charcot Neuroarthropathy Increased risk of falls and fractures	At risk of foot ulcers and secondary ischaemia and infection

Table 1.2 shows the key differences in the pathological subtypes of distal symmetrical DN. Table collated using information from review of literature by Vinik et al. (190).

Obesity, independent of T2DM has been associated with neuropathy (197). A recent large population-based study from China (n=4002) showed that although hyperglycaemia showed the strongest association with peripheral neuropathy (odds ratio [OR] 2.06; 95% confidence interval [CI] 1.77-3.80), obesity also increased risk (OR 1.09; 95% CI 1.02-1.18) independent of dysglycaemia. Furthermore, in this study, even in patients without diabetes, the risk of distal symmetrical neuropathy was higher when there was a greater number of components of the metabolic syndrome present. Similarly, in patients with diabetes, a greater number of metabolic syndrome factors conferred a higher risk of peripheral neuropathy which may suggest that an obese person with diabetes is more likely to get DN (197).

Correspondingly, Herman *et al.* showed that obese patients showed clinical manifestations of small nerve fibre dysfunction independent of hyperglycaemia and hyperinsulinaemia (198).

There is little evidence about the effects of bariatric surgery on DN. A previous retrospective study suggested an incidence of 16% of peripheral neuropathy after bariatric surgery (199). The authors hypothesised that this was probably precipitated by malnutrition as non-attendance at a nutritional clinic follow-up appointment was a predictive factor (199). Interestingly, the jejuno-ileal bypass procedure was also a predictive factor (199) so the findings may not be as applicable in the modern day as this procedure is not commonly performed anymore. Conversely, another study (n=20) showed that there were significant improvements in the neuropathy symptom and disability scores 6 months after bariatric surgery. Neuropathic improvements were manifest by improved vibration perception and ankle reflexes as opposed to pain and temperature sensation, implying large fibre benefits (200). Contrastingly, Miras *et al.* did not find any signficant improvements in nerve conduction velocities or amplitudes twelve months post bariatric surgery (178).

1.7.3.1 Corneal Confocal Microscopy

The cornea is a highly innervated structure (201) therefore appropriate imaging of this can provide vital information about underlying neuropathic disease. Corneal confocal microscopy (CCM) is a procedure which has had increasing diagnostic utility in a number of peripheral neuropathies, including DN (202). This method detects small fibre nerve damage which allows for the earlier detection of neuropathy-related peripheral nerve changes (203). Advantages include the non-invasive nature of the testing, reproducibility of imaging and as above, relative sensitivity (202). This is further evident by a previous study showing DN changes in peripheral nerves in apparently healthy patients without an established diagnosis of diabetes (though with HbA1c levels tending towards the upper limit of normal) suggestive of the ability of CCM to detect sub-clinical and pre-diabetic nerve insult (204). Furthermore, CCM has been validated to quantify the extent of small fibre nerve damage in a comparable manner to the pre-existing ‘gold-standard’ intra-

epidermal nerve fibre density which is measured after nerve sampling by invasive skin biopsy (203). CCM does incur considerable short-term cost and requires specialised equipment and operator training which can provide limitations for its use (202). The speed at which acquired images are analysed can also provide an impediment to use (202).

CCM has also been used to show post-intervention changes in nerves as has been the case with DN after addressing hyperglycaemia (205) as well with simultaneous kidney and pancreas transplantation when there was an improvement in corneal nerve fibre density (206). Considering this further, CCM provides an excellent means by which DN can be studied effectively firstly in certain disease contexts (for example obesity) and secondly to assess the influence of any intervention (for example bariatric surgery).

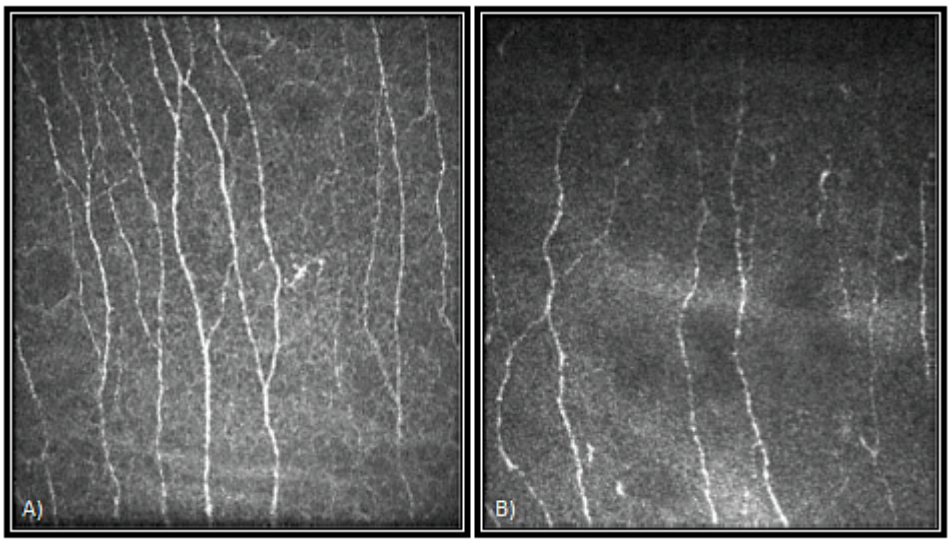


Figure 1.11. Example CCM image from A) a healthy volunteer and B) a patient with diabetes. Adapted from Wang et al. (207). The patient with diabetes has less visible nerve fibres (depicted by white lines).

1.7.4. Erectile dysfunction

Obesity and diabetes are both associated with increased risk of erectile dysfunction (208, 209). Among men with erectile dysfunction, the reported prevalence of overweight and obesity is up to 79% (210). In addition, the presence of diabetes has been associated with a three-fold increase in risk of erectile dysfunction (211).

Normal erectile function is dependent upon a combination of complex vascular, neural, hormonal and psychological factors (212). Sexual stimulation triggers physiological activation of the parasympathetic nervous system and release of nitric oxide which exerts a cascade of vascular effects culminating in increased penile blood flow and increase in pressure within the corpora cavernosa leading to penile erection (213, 214). Testosterone has a role in modulating cavernosal nerve, smooth muscle and vascular endothelial function (215-217).

Microvascular disease in diabetes comprises the ischaemic insult involving the distal circulation and peripheral neuropathy. Small autonomic nerve fibres are fundamental to normal erectile function and specifically, erectile dysfunction has been highly correlated with small nerve fibre dysfunction assessed using thermal sensory testing (218, 219). Insulin resistance and adiposity are closely linked with the state of chronic low-grade inflammation that is the hallmark of obesity and metabolic syndrome (220, 221). The underlying inflammatory process and hyperglycaemia results in endothelial dysfunction leading to impairment in nitric oxide release and activity (222, 223). Given the central role of neural and vascular factors in the underlying pathophysiology, erectile dysfunction is therefore a good reflection of burden of microvascular disease in diabetes and obesity.

Hypogonadism is also common in obesity with a reported prevalence rate of 53.4% (95% CI 47.9–56.9) in the Hypogonadism in Males (HIM) study (224). In this study involving 130 primary care practices across the United States, BMI was identified as the most significant differentiating factor between hypogonadal and eugonadal patients. Insulin resistance has been proposed to negatively impact on testosterone secretion from Leydig cells, with a previous study showing correlation between stimulated testosterone levels and insulin sensitivity scores (225). Furthermore,

aromatisation of androgens within adipose tissue and the resultant accumulation of oestradiol can lead to suppression of luteinising hormone production via negative feedback. In support of this theory, administration of aromatase inhibitors has been found to normalise testosterone levels in obesity (226).

Despite the complex pathophysiology underlying erectile dysfunction in obesity and diabetes, low testosterone is often the main focus in clinical practice. Current guidelines recommend testosterone replacement in men with androgen deficiency with the aim of improving symptoms and sexual function (227). However, the evidence supporting the benefits of testosterone replacement on sexual function, particularly in men with obesity, is inconsistent (228-231).

1.7.5. Bariatric Surgery influence on incident microvascular disease

Bariatric surgery has been shown to reduce incident microvascular disease. Coleman *et al.* in a large (n=4863) health-record based retrospective study showed that over a 10-year period, bariatric surgery reduced the incidence of microvascular disease by 29%. Remarkably, similar to other interventional trials of diabetes (232, 233), the authors showed a 'legacy effect' of surgery; for patients who suffered from relapse of their T2DM, the period spent in remission offered cumulative protection from microvascular complications (234). This study relied predominantly on diagnostic 'read-codes' which therefore provides a major limitation when regarding the accuracy of its findings (234). Interestingly, the main microvascular benefit in this study was found in relation to retinopathy (36% reduction in incidence over a 7-year period) which is contrary to a recent meta-analysis of DR post bariatric surgery (180).

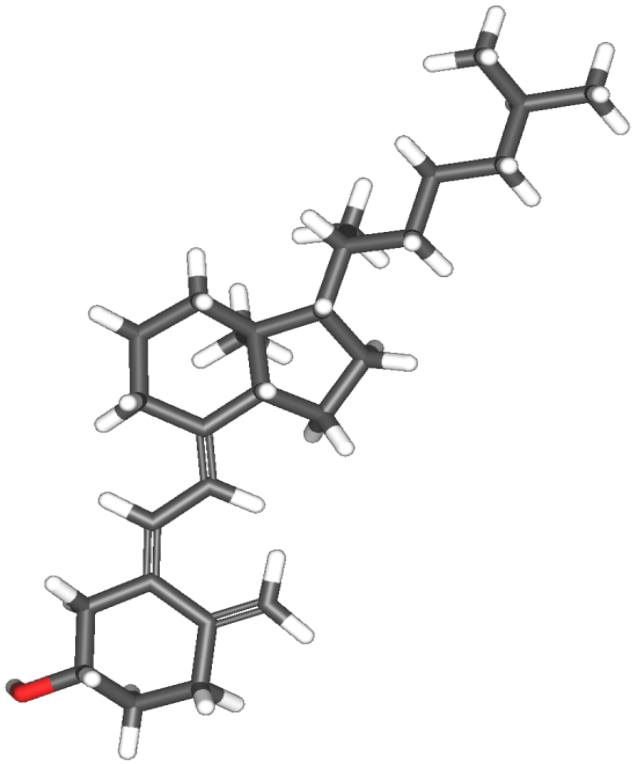
Another large retrospective study by Johnson *et al.* reported that 5-year incident microvascular disease (defined as laser eye surgery or blindness, dialysis treatment and lower limb amputation as proxies for DR, DKD and DN respectively) was reduced by 78% after bariatric surgery (235). A recent meta-analysis by Sheng *et al.* analysing approximately 17000 patients (in 10 studies with at least 5 years' follow-up data) revealed a reduction in incident microvascular disease of 79%. Of note, there was marked heterogeneity in the studies which included differences in the exact definitions of microvascular disease (236).

Results from the longitudinal SOS comparing the incidence of microvascular disease in 2010 patients who underwent bariatric surgery to 2037matched controls showed that there was a marked reduction in the incidence of microvascular disease 15 years post-operatively. This was especially evident in patients with prediabetes at the initial visit (OR 0.18; 95% CI 0.11-0.30). The reductions in incidence of microvascular disease were 46% and 37% respectively for those with established diabetes and euglycaemic patients at baseline. Importantly, this study also relied on data stored in the Swedish National Patient Register and was therefore prone to crude definitions and as well as inaccuracy of data (237).

1.8 Obesity, vitamin D deficiency and bariatric surgery

1.8.1 The systemic effects of vitamin D

Vitamin D is one of the fat-soluble vitamins and has a primary function in calcium homeostasis and bone metabolism (238). In addition, there has been gathering evidence of a role in immune disorders, diabetes, neoplasia and neuropsychiatric disease (238) (Figure 1.12).



The multi-faceted effects of Vitamin D

- **Bone and Calcium Homeostasis**
- Immune System
- Diabetes
- Neoplasia
- Neuropsychiatry

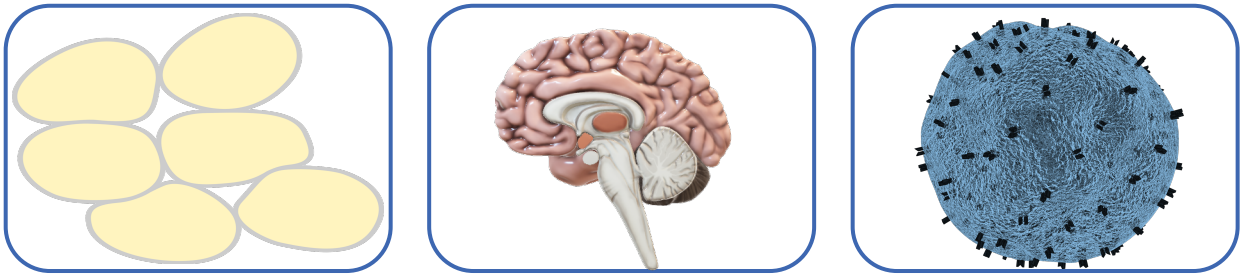
Figure 1.12. Chemical structure of vitamin D_3 (cholecalciferol) and a list of possible functional and pathological roles.

Chemical structure figure copied, under current permissions [CC BY-SA from https://upload.wikimedia.org/wikipedia/commons/3/3d/Cholecalciferol-3d.png. Different roles of vitamin D derived from review in Wang et al. (237).

1.8.2 Vitamin D and Obesity: a bi-directional relationship?

Obesity has been linked to vitamin D deficiency. Indeed, a previous meta-analysis of 21 studies comparing obese persons to normal weight individuals, the prevalence ratio of vitamin D deficiency was 35% higher in obesity (95% CI 21-50% increase in prevalence ratio) (239). The limitation of this otherwise thorough meta-analysis was that the authors were unable to correct for differing dietary habits and seasonal variations in the studies (239). Furthermore, in another study where both obese patients and normal-weight individuals were subjected to similar amounts of sunlight (a key factor in vitamin d synthesis/activation), there was almost a 2-fold difference in how much serum vitamin d levels increased (240). The obese patients showed lower vitamin D levels even when accounting for vitamin d precursor levels in the skin (240).

The relationship between vitamin D and obesity has been postulated as being bi-directional, especially due to the expression of vitamin D receptors in adipose tissue (241). On the one hand, obesity is thought to be a contributory factor in vitamin D deficiency and on the other, vitamin D deficiency is thought to increase the risk of adiposity, obesity and insulin resistance (242). It has been hypothesised that adipose tissue itself has a local use for vitamin D (given the receptor expression) (241). Additionally, there is the possibility that vitamin D undergoes breakdown by adipose tissue before its hydroxylation (to activate it) in the liver can occur (240). The mechanisms by which vitamin D deficiency can lead to obesity are summarised below (Figure 1.13). Briefly, largely through unknown pathways, vitamin D deficiency can lead to reductions in lipolysis and promote adipogenesis which leads to adipocyte hypertrophy (242). Furthermore, a state of chronic inflammation imparted by vitamin D deficiency can lead to an increased risk of obesity (242). It has also been speculated that vitamin D deficiency can increase the hunger impulse which in turn leads to a greater tendency towards obesity (242).



Adipose Tissue

Increase in lipogenesis & reduction in lipolysis leads to fat accumulation

Central Nervous System

Increases in hunger sensation

Immune System

Increased amount of cytokines leads to chronic inflammation

Figure 1.13. Possible mechanisms by which vitamin D deficiency increases risk of obesity.

Information for figure schematic obtained from review article by Cândido & Bresnan (242).

1.8.3 Bariatric Surgery and Vitamin D Deficiency

Bariatric surgery can lead to deficiencies in vitamin D amongst other micronutrients (243-246). Previous meta-analyses have shown an increased risk of fractures and therefore a detrimental effect on bones induced by bariatric surgery (247, 248) with deficiency in vitamin D being a contributory factor (249). Another systematic review (51 observational studies) showed that mean vitamin D levels were ≤ 50 nmol/L (20 ng/mL) in a third of studies and despite vitamin D supplementation after bariatric surgery the average vitamin D levels remained ≤ 75 nmol/L (30 ng/mL) in the majority of studies (246). Importantly, Li *et al.* showed in a meta-analysis of 12 studies (1285 patients) that vitamin D supplementation (>800 IU/day) reduced the risk of vitamin D deficiency 12 months post-operatively (250). In this meta-analysis, a dose of <800 IU/day was not protective against vitamin D deficiency one-year post-surgery (250). The British Obesity & Metabolic Surgery Society clinical guidelines recommend that patients take a combination of ≥ 800 IU vitamin D and 1000–1200 mg calcium daily following bariatric surgery (244).

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Chapter 2: Hypotheses and Aims

Obesity is associated with a multitude of metabolic complications including effects on CVD risk, insulin resistance, augmentation of microvascular disease and an adverse metabolic bone health profile. Bariatric surgery will cause improvements in these risk factors. In obese people, it is important to understand i) the respective contribution of different metabolic factors to the development of obesity-related complications and ii) how the most effective treatment for obesity, bariatric surgery, reduces the co-morbid disease burden.

Aims:

i. To assess the effect of bariatric surgery on insulin resistance and insulin secretion and use this as a model to understand the basis of the Reaven hypothesis. To determine the relationships between changes in insulin resistance and insulin secretion with changes in sdLDL and interleukin-6 (IL-6).
ii. To establish the impact of bariatric surgery on HDL functionality and elucidate factors that may affect any post-operative changes in HDL function.
iii. To assess the impact of bariatric surgery on anti-apoA-1 IgG levels and seropositivity.
iv. To quantitatively assess the impact of bariatric surgery on the microvascular complications of T2DM.
v. To examine the relationship between small fibre neuropathy, sex hormones and sexual dysfunction in men with severe obesity.
vi. To study the pre- and post-operative prevalence of vitamin D deficiency in obese patients who undergo bariatric surgery.

Chapter 3: Methodology

3.1 Preface

This chapter covers the methodology used for the results chapters. It provides a detailed description of the study design and assessments undertaken. With this thesis being presented in alternative format, this chapter overlaps with the methods section within each individual results chapter.

3.2 Study design

This thesis was formed using results generated from two studies and the study design are described below.

3.2.1. Prospective observational study

The first study was a prospective observational study of patients with obesity who underwent bariatric surgery, and this forms the majority of this thesis. Patients were recruited from the pre-operative clinic at the tier 4 weight management centre at Salford Royal NHS Foundation Trust (Salford, UK). Study assessments were undertaken at the Manchester National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK) at baseline, 6 months, and 12 months after bariatric surgery. Study assessments include medical history, anthropometric measurements, neuropathy assessment, sexual function assessment where appropriate, and laboratory measurements. Further details on each of the assessments are provided below.

Inclusion criteria:

- All patients with obesity being offered bariatric surgery under the United Kindgom National Health Service (NHS).
- Age above 18 years
- Able to provide informed consent

The BMI criteria for bariatric surgery is in accordance with the National Institute of Health and Care Excellence (NICE) guidance: BMI of 40 kg/m^2 or more, or BMI

between 35 kg/m^2 and 40 kg/m^2 in the presence of other significant weight-related disease (1).

Exclusion criteria:

- Active infection
- Human immunodeficiency virus infection
- Recent acute coronary syndrome (within 6 months)
- History of malignancy and chemotherapy
- Haematological disorder
- Autoimmune disease
- History of immunotherapy
- Participation in other interventional research trial
- Non-statin lipid-lowering therapy

Additional exclusion criteria for assessment of neuropathy:

- History of corneal trauma or surgery
- Recent cataract surgery (within 12 months)
- History of ocular disease, infection or inflammation
- History of non-diabetic neuropathy
- Active diabetic foot ulceration or infection

Additional exclusion criteria for assessment of sexual function:

- History of cardiovascular disease
- History of pituitary, adrenal or testicular disorder
- Current therapy for erectile dysfunction
- Primary hypogonadism (luteinising hormone >9.4 U/L)
- Medications known to affect androgen levels

3.2.2 Retrospective observational cohort analysis

The second study cohort was a retrospective observational cohort analysis of longitudinal data on vitamin D and related markers in patients who underwent bariatric surgery. Data over a four-year follow-up period was collected from the

Salford Royal NHS Foundation Trust electronic patient record. All patients with a minimum of 12 months post-operative follow-up were included. Data collected included patient demographics, weight and BMI, laboratory results including total vitamin D (25-hydroxyvitamin D), phosphate, alkaline phosphatase (ALP), parathyroid hormone (PTH), albumin, total and adjusted calcium, and HbA1c.

3.3 Ethical approval

The prospective observational study on bariatric surgery was approved by the Greater Manchester Central Research and Ethics Committee (REC Reference: 11/NW/0731), the Manchester University National Health Service (NHS) Foundation Trust Research and Development office, and the Scientific Advisory Board of the Manchester National Institute of Health Research/Wellcome Trust Clinical Research Facility. The study was undertaken in accordance with the principles of the 1964 Helsinki declaration. Written informed consent was obtained from all study participants prior to entry into the study with study literature provided at least 24 hours prior to consent. Participant information sheets and consent forms are attached in the appendix of this thesis.
The retrospective observational cohort study on vitamin D status in patients who underwent bariatric surgery was approved by the Clinical Audit Department of Salford Royal NHS Foundation Trust.

3.4 Study assessments

The following study assessments were undertaken before, and at 6 months and 12 months after bariatric surgery.

3.4.1 Medical history

Patient demographics, co-morbidities, and medications were collected during each study visit..

Presence of T2DM was determined via the medical history and a further HbA1c measurement at baseline was also used to identify patients with undiagnosed T2DM defined as HbA1c ≥ 48 mmol/mol.

3.4.2 Anthropometric measurements

Measurements of height, weight and waist circumference were obtained at all study time points. BMI was calculated using the standard equation: weight in kg/(height in m)^2.

Assessment of blood pressure was undertaken using an automated blood pressure device with appropriate cuff size. Three measurements systolic and diastolic blood pressure were taken and the average of the last two readings was calculated.

3.4.3 Neuropathy assessment

3.4.3.1 Assessment of symptoms

The neuropathy symptom profile (NSP) was used for assessment of neuropathy-related symptoms. It is a 38-item questionnaire covering symptoms relating to the motor, sensory and autonomic system. The maximum score of 38 equates to the most severe neuropathy-related symptoms whereas a score of 0 represents an absence of symptoms.

3.4.3.2 Clinical examination

Patients had sensorimotor assessment of their lower limbs in order to calculate the Neuropathy Disability Score (NDS; Table 3.1) which has a maximum score of 10. Both right and left feet were assessed individually using a single-blinded fashion (patient asked to close their eyes to remove visual bias during assessment). Clinical neuropathy examination comprised of 4 domains:

- Pain sensation as measured by pin-prick assessment of both feet (distal to proximal assessment) using Neurotips™ (Owen Mumford Ltd, Oxford, United Kingdom). Pin-prick sensation on feet compared to site on forearm as reference point (provided the patient had no known upper limb neuropathic disease).

• Vibration assessment (starting distally and moving proximally as needed on bony prominences) was performed using a 128-Hz tuning fork on both feet.

• Temperature sensation was measured using warm and cold metal rods. The purpose of this exam was for gross discrimination between warm and cold temperature sensation and the distinction between rods was assessed by the investigator beforehand after pre-heating one of the rods and cooling the other using warm and cold water respectively.

• Achilles (ankle) reflex jerk. This was performed with the patient kneeling on one knee with the foot 'hanging' at the bedside and asking the patient to remain in a relaxed state. A tendon hammer was used to assess the reflex and if the reflex was difficult to obtain, reinforcement technique was used (asking patient to clench hands together) to unmask difficult reflex.

Table 3.1. Neuropathy Disability Score Assessment Scoring System

		Right			**Left**	
Test	**Normal**	**Reinforcement (Achilles reflex only)**	**Abnormal**	**Normal**	**Reinforcement (Achilles reflex only)**	**Abnormal**
Pain (pinprick)	0		1	0		1
Vibration (tuning fork)	0		1	0		1
Temperature (hot and cold rods)	0		1	0		1
Achilles Reflex	0	1	2	0	1	2
				Total NDS		**/10**

The Neuropathy Disability Score (NDS) is calculated by assessments in four main clinical domains on each foot. Each assessment is scored individually and then a total score (out of 10) is calculated.

Following NDS measurement, severity of neuropathy is graded as follows:

- *NDS 0-2: Normal*
- *NDS 3-5: Mild Peripheral Neuropathy*
- *NDS 6-8: Moderate Peripheral Neuropathy*
- *NDS 9-10: Severe Peripheral Neuropathy*

3.4.3.3 Quantitative sensory testing (QST)

QST was performed on each patient using a MEDOC TSA II Neurosensory Analyser (Medoc, Ramat Yishai, Israel) to determine thresholds for cold sensitivity, warm sensitivity, cold induced pain and warm induced pain. Equipment consisted of a temperature probe (thermode) connected to a main unit which controls the temperature of the thermode according to stimuli received. A 'patient response unit' (in the form of a computer 'mouse') allowed participant responses to different test stimuli. The unit was connected to a Windows based laptop with pre-installed manufacturer supplied software.

For the testing, the thermode was applied to the dorsum of the patient's non-dominant foot (S1 dermatome area) and the patient was given the Patient Response Unit and asked to indicate (by clicking the mouse) the following 4 parameters:

- **Cold Sensation (CS):** The first moment the thermode felt cold
- **Warm Sensation (WS):** The first moment the thermode felt warm
- **Cold Induced Pain (CIP):** First moment of cold induced discomfort
- **Warmth Induced Pain (WIP):** First moment of warm induced discomfort

The respective temperature for each of these thresholds was recorded by the investigator supervising the test. The lower the threshold to sense a cold temperature sensation suggested a greater degree of cold insensitivity; conversely the higher the threshold to sense a warm temperature sensation suggested a greater degree of heat insensitivity.

Vibration perception testing was done using a neurothesiometer probe (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.) which measures a range from 0-50 volts. The mean reading of 3 separate readings per foot were used as the measurements for each patient. The higher the voltage stimulus required to produce a vibratory sensation, the less perceptive the foot was deemed to be to this sensory modality.

3.4.3.4 Corneal confocal microscopy

Patients underwent examination with a Heidelberg HRTII Rostock Cornea Module confocal microscope (Heidelberg Eye Explore, Heidelberg Engineering GmBH and Germany). The refraction of the objective lens of the corneal confocal microscope was set at +12 dioptres with the camera being set at the lowest possible position. The objective lens of the confocal microscope was disinfected using a medi-swab (isopropyl alcohol 70% v/v, Swabs). Viscotears liquid gel (carbomer 980, 0.2%, Novartis, UK) was applied onto the tip of the lens and this drop was covered using a sterile TomoCap. The drop of Viscotears served as a coupling medium and covered the gap between the lens and the TomoCap. The camera was then adjusted for both depth and resolution to allow for optimal image acquisition.

Prior to testing, patient preparation included detailed instructions as well as using anaesthetic drops (0.4% Benoxinate Hydrochloride, Chauvin Pharmaceuticals Ltd., Essex, UK) and lubricant drops (Viscotears, Carbomer 980, 0.2%, Novartis, UK) in both eyes. The examiner aligned the objective lens on the central cornea while the patient looked directly into the fixation light of the instrument. The image acquisition was guided by 'real-time' views of the cornea on a laptop connected to the microscope which had pre-installed image acquisition software on it. Using the 'section' mode, several images of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backwards and forwards. The distance between each image was approximately 1 μm therefore allowing for ten images at a depth of 10 μm. Following appropriate image acquisition and cessation of the examination, the TomoCap was removed and the equipment was disinfected with a further sterile medi-swab (isopropyl alcohol 70% v/v, Swabs). On average, six high quality images of Bowman's layer (at the level of the sub-basal nerve plexus) were selected per patient for further image analysis.

Corneal nerve parameters were quantified manually using CCMetrics (The University of Manchester, UK), a form of highly specialised software. For the purposes of the study, the following parameters were used as indicators of corneal nerve fibre damage and repair:

- Corneal nerve fibre density (CNFD) – major nerves/mm^2 corneal tissue.

- Corneal nerve branch density (CNBD) – nerve branches/mm^2 corneal tissue.
- Corneal nerve fibre length (CNFL) – length of nerves/mm^2 corneal tissue

3.4.3.5 Nerve conduction study

Nerve conduction study was undertaken by a consultant neurophysiologist (Dr Andrew Marshall) using a Dantec “Keypoint” System (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator for maintenance of limb temperature between 32 to 35 °C. Silver-silver chloride surface electrodes were used at defined anatomical landmarks assessment of motor nerve and recordings for sural sensory nerve was taken using antidromic stimulation over a distance of 140 mm.

The stimulus strength was increased until maximal response was obtained and the further increased by 10 to 15% to produce a supramaximal response. Both sensory and motor amplitudes were measured from baseline to the negative peak. The sensory nerve action potential is reported to the nearest 0.1 μV and the motor action potential reported to the nearest 0.1 mV.

The following parameters were measured:

- Sural nerve amplitude
- Sural nerve conduction velocity
- Sural nerve latency
- Peroneal nerve amplitude
- Peroneal nerve conduction velocity
- Peroneal nerve latency
- Radial nerve amplitude
- Radial nerve velocity

3.4.5 Sexual function assessment

Sexual function was assessed using the European Male Ageing Study Sexual Function Questionnaire (EMAS-SFQ) (2) (see appendix). The EMAS-SFQ is a 20-item questionnaire validated for its retest and internal consistency reliability in middle-aged and elderly men between the ages of 40 and 75 years. For this study, we selected three sexual symptoms most associated with hypogonadism (3) for assessment of sexual function: erectile function, frequency of sexual thoughts and frequency of morning erections. Established cut-offs were used to determine if patients are symptomatic for each of the three sexual symptoms (Table 3.1). The definitions of asymptomatic and symptomatic response categories are based on validated published criteria (3, 4).

Table 3.2. Questions regarding sexual symptoms within the EMAS-SFQ and definitions of asymptomatic and symptomatic categories.

	Asymptomatic	**Symptomatic**
Were you able to get and keep an erection sufficient for sexual intercourse?	Usually or always	Never or sometimes
How often did you think about sex?	Once a week or more	2–3 times in the past month
How frequently did you awaken with a full erection in the past month?	2–3 times in the past month	≤1 time in the past month

Reproduced from Wu et al. (3)

3.4.6 Laboratory measurements

Venous blood samples were obtained from patients between 0800 and 1100 following an overnight fast of at least 12 hours. All laboratory measurements were undertaken at the end of the study apart from HbA1c, serum creatinine, bone profile, and vitamin D which were measured on the day of study visit.

3.4.6.1 Separation of serum and plasma

Serum and EDTA-plasma were isolated by centrifugation at 3300 rpm for 15 minutes at 4°C within 2 hours of collection. Serum and plasma aliquots were stored at -20 °C or -80°C until analysed. Each serum or plasma aliquot underwent one freeze-thaw cycle only.

3.4.6.2 Glycated haemoglobin

HbA1c was measured on the day of study visit by HPLC using a VARIANT II Turbo Haemoglobin Testing System (Bio-Rad Laboratories, Hemel Hempstead, UK) in the Department of Biochemistry, Manchester University NHS Foundation Trust.

3.4.6.3 Total cholesterol

Total cholesterol was measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) method. 3 μl of sample was added to 20 μl of H_2O and 250 μl of reagent. Cholesterol is oxidised by cholesterol oxidase following enzymatic hydrolysis by cholesterol esterase. The hydrogen peroxide released reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form quinoneimine. The cholesterol concentration correlates with increase in absorption at 500 nm, which is measured using Cobas Mira analyser (Horiba ABX Diagnostics, Northampton, UK). The intra- and inter-assay coefficients of variation (CV) were 2.7% and 3.4% respectively.

3.4.6.4 Triglycerides

Triglycerides were measured using the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) method. 3 μl of sample was added to 10 μl of H^2O and 290 μl of reagent. Hydrogen peroxide is released following oxidation by glycerol-3-phospate oxidase and quinoeimine in generated from 4-aminoantipyrine and phenol in the presence of peroxidase. Triglyceride concentration correlates with the increase in absorbance at 500 nm, which is measured using Cobas Mira

analyser (Horiba ABX Diagnostics, Northampton, UK). The intra- and inter-assay CV were 3.3% and 3.5% respectively.

3.4.6.5 High-density lipoprotein cholesterol

HDL-C was measured by a direct homogeneous method. 3 µl of sample was added to 50 µl of H_2O, 250 µl of reagent 1 (*N*,*N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonphonic acid, *N*-(2-hydroxy-3-Sulfopropyl)-3,5-dimethoxyaniline, sodium salt, cholesterol esterase, cholesterol oxidase, catalase and ascorbate oxidase), 83 µl of reagent 2 (*N*,*N*-Bis(2-hydroxyethyly)-2-aminoethanesulphonic acid, 4-aminoantipyrine, horse radish peroxidase, sodium azide and surfactants). Polyethylene glycol (PEG)-modified cholesterol esterase breaks down HDL-cholesterol esters into free cholesterol and fatty acids. Cholesterol is then oxidised by cholesterol oxidase and hydrogen peroxide is generated which reacts with 4-aminoantipyrine and *N*-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline. The concentration of HDL-cholesterol correlates with the increase in absorbance at 600 nm, which is measured using Cobas Mira analyser (Horiba ABX Diagnostics, Northampton, UK). The intra- and inter-assay CV were 1.2 and 0.9% respectively.

3.4.6.6 Low-density lipoprotein cholesterol

LDL-C levels were derived from total cholesterol, triglyceride, and HDL-cholesterol using the Friedewald formula (5). This formula was used only when serum triglyceride did not exceed 4.5 mmol/l.

LDL-C (mmol/l) = total cholesterol (mmol/l) – HDL-C (mmol/l) – (triglyceride (mmol/l) / 2.19)

3.4.6.7 Apolipoprotein B

ApoB was measured using immunoturbidimetric immunoassay. 13 µl of sample was added to 30 µl of H_2O, 200 µl of PBS polymer solution, 16.7 µl of anti- human apoB antibody, and 53.3 µl of PBS. The signal generated on turbidimetry was measured at 340nm using Cobas Mira analyser (Horiba ABX Diagnostics, Northampton, UK) and

this correlates with the concentration of ApoB. The intra- and inter-assay CV were 2.2 and 2.6% respectively.

3.4.6.8 Small-dense LDL (sdLDL) apoB

Small-dense LDL ApoB (LDL particles of density >1.044g/ml) was isolated from plasma and ultracentrifuged at 100,000 rpm (435,680 × g) for 5 hours at 4°C using a Beckman Optima TLX bench top ultracentrifuge fitted with TLA 120.2 fixed angle rotor (Beckman Coulter UK) (6). ApoB in sdLDL was then determined using immunoturbidimetric assay with the generated signal measured using Cobas Mira analyser (Horiba ABX Diagnostics, Northampton, UK). sdLDL is therefore expressed in terms of the plasma concentration of its ApoB component.

3.4.6.9 Apolipoprotein A-1

ApoA-1 was measured using immunoturbidimetric assay. 7 µl of sample was added to 60 µl H_2O, 200 µl of PBS polymer solution, 23.3 µl of purified immunoglobulins from rabbit antiserum (apoA-I from human HDL immunogen) and 46.7 µl PBS. The signal generated on turbidimetry is measured at 340 nm after 10 and 15 minutes using Cobas Mira analyser (Horiba ABX Diagnostics, Northampton, UK) and this correlates directly with the concentration of apoA-I.

3.4.6.10 Anti-apolipoprotein A-1 IgG

Anti-apoA-1 IgG autoantibodies levels and positivity were determined using methods established at the Department of Genetics and Laboratory Medicine, Geneva University Hospital (Geneva, Switzerland) (7-9).
Maxisorb plates (Nunc, Glostrup, Denmark) were coated with purified human-derived delipidated apoA-1 (20 µg/ml; 50 µl/well) for 1 hour at 37°C. After 3 washes, all wells were blocked for 1 hour with 2% bovine serum albumin (BSA) in a phosphate buffer solution (PBS) at 37°C. Samples were then diluted to 1:50 in PBS/BSA 2% solution and incubated for 60 minutes. Samples at the same dilution were also added to non-coated wells to assess individual non-specific binding. After 6 washes, 50 µl of signal

antibody (alkaline phosphatase-conjugated anti-human IgG; Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:1000 in PBS/BSA 2% solution was added to each well and incubated for 1 hour at 37 °C. After a further 6 washes, 50 µl of phosphatase substrate *p*-nitrophenyl phosphate disodium (Sigma-Aldrich, St Louis, MO, USA) dissolved in diethanolamine buffer (pH 9.8) was added. Following an incubation period of 20 minutes at 37°C, each sample was tested in duplicates and optical density (OD) was determined at 405nm (Molecular Devices™ Versa Max, Sunny Vale, CA, USA). The corresponding non-specific binding was subtracted from mean absorbance for each sample. The specificity of detection was confirmed by conventional saturation tests.

The upper reference range used as a cut off for anti-apoA-1 positivity was derived from the 97.5th percentile of reference population for 140 healthy blood donors and this corresponded with an OD cut off of 0.64. An index consisting the ratio between sample OD and the positive control OD expressed as a percentage is further calculated to minimise the impact of inter-assay variation. The index value of 37% corresponded with the 97.5th percentile of the normal distribution. Samples with an absorbance value >0.64 OD and an index value ≥37% were considered positive for elevated anti-apoA-1 IgG levels.

3.4.6.11 Cholesterol efflux capacity

Cholesterol efflux capacity was measured using a previously validated method (10). J774A.1 macrophage cells were cultured in RPMI 1640 medium containing 10% FBS, 100 IU/ml Penicillin and 100 g/ml Streptomycin at 37 ºC in a humidified incubator with 5% carbon dioxide for 3 days. Cells were washed with Hanks solution and collected by centrifugation at 1500 rpm for 5 minutes. The infranatant was removed and cells were suspended in new media (0.4% trypan blue solution) to check for viability and cell count. The cells were then plated at a final concentration of 5 x 10^5 cells/ml (1 ml per well) and incubated for 24 hours. Plated cells were then washed and incubated with 0.2 µCi of radiolabelled ^{3}H-cholesterol per ml of RPMI 1640 medium containing 0.2% BSA, 100 IU/ml Penicllin and 100 g/ml Streptomycin in a humidified incubator with 5% carbon dioxide for 24 hours. ABCA1 transporter

was upregulated using medium containing 0.3 mM C-AMP (8-(4-Chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt) for 4 hours. ApoB-depleted serum was prepared using 20% polyethylene glycol solution and precipitate removed after 20 minutes by centrifugation at 10,000 rpm at 4 ºC for 30 minutes. 2.8% apoB-depleted serum (28 µl apoB-depleted serum and 972 µl media) was then added to the washed cells. Cell media was then collected after 4 hours of incubation, dissolved in 0.5 ml 0.2 NaOH, and 2ml of liquid scintillator added and mixed thoroughly. Radioactivity was determined using liquid scintillation analyser Packard TRI-CARB 2100 TR (Perkin Elmer, Massachusetts, USA).

$$\text{Cholesterol efflux (\%)} = \frac{\text{Radioactivity in medium}}{\text{Radioactivity in cell + radioactivity in medium}} \times 100$$

3.4.6.12 Paraoxonase-1 activity

Serum PON1 activity was determined using a semi-automated micro-titre plate method using paraoxon (*O,O*-Diethyl *O*-(4-nitrophenyl)phosphate) (Sigma Chemical, Poole, UK) as a substrate. 30 µl of sample was added to cryogenic tubes which were then loaded into Cobas Mira analyser (Horiba ABX Diagnostics, Northampton, UK) and Paraoxone Stock Solution (36.36 µl paraoxon and 40 ml assay buffer to give 5.5 mmol/l paraoxon) added to the substrate container. Using the PON1 activity measuring programme, a kinetic model is employed to calculate the change in OD at 405 nm per unit time. The intra- and inter-assay CV were 4.9 and 5.3% respectively.

PON1 activity (nmol / ml / min) = OD / min x 2057

3.4.6.13 Cholesteryl ester transfer protein activity

CETP enzyme activity was measured using Abcam fluorometric assay (Abcam, Cambridge, UK). The fluorescence intensity (excitation λ = 465nm, emission λ = 535nm) was measured using BioTek FLx800 microplate fluorescence reader (Fisher Scientific, New Hampsire, USA) to generate the standard curve. Sample and control were added in duplicate to each well followed by the reaction mixture comprising of 10 µl of Donor Molecule Solution, 10 µl of Acceptor Molecule solution, and 20 µl of CETP assay Buffer Solution. Following an incubation period of 1 hour at 37 ºC, the fluorescence intensity (excitation λ = 465nm, emission λ = 535nm) was determined.

The intensity was directly proportional to the activity of CETP enzyme. Given the fluorescent self-quenched lipid from the donor molecules was transferred to the acceptor molecules by the action of CETP enzyme present in the sample, CETP activity is represented by the measured fluorescence intensity. The intra- and inter-assay CV were 4.7 and 8.6% respectively.

3.4.6.14 Myeloperoxidase (MPO) mass

MPO mass is determined using an in-house antibody sandwich ELISA technique. 100 µl of Capture Antibody (lyophilized rat anti-human MPO (R&D Systems Europe, Abingdon, UK) in PBS) was added to each well and incubated overnight at room temperature. After 3 washes (wash buffer 0.05% (v/v) Tween-20 in PBS), 300 µl of Reagent Diluent (BSA in PBS and *dd* H_20) was added and incubated for 60 minutes at room temperature. 100 µl of samples and MPO standards (R&D Systems, Abingdon, UK) were then added and incubated for 2 hours at room temperature. This is followed by the addition of 100 µl of Detection Antibody (lyophilized biotinylated goat anti-human MPO (R&D Systems Europe, Abingdon, UK) with Reagent diluent) and further 1 hour of incubation at room temperature. Streptavidin-HRP (R&D Systems Europe, Abingdon, UK) is then added to each well and incubated for 30 minutes in the dark at room temperature. Each of the wells were washed 3 times in between each of the above steps. 100 µl of TMB was then added to each well at 20 second intervals and incubated for 15 to 30 minutes at room temperature, following which 50 µl of Stop Solution (1M sulphuric acid) was added at 20 second intervals. OD of each well is determined using a microplate reader set to 630 nm and the concentration is calculated from the standard curve.

3.4.6.15 High-sensitivity C-reactive protein

High-sensitivity CRP (hsCRP) was measured using immunoturbidimetric assay. Sample was added to Assay Buffer (Glycine, sodium chloride, sodium EDTA disodium salt dihydrate and BSA) and Antibody Reagent (Latex particles coated with antibody to CRP). The signal generated on turbidimetry is measured at 570 nm after

10 and 15 minutes using Cobas Mira auto-analyser (Horiba ABX-UK, Northampton, UK).

3.4.6.16 Tumour necrosis factor alpha

TNF-α was measured using quantitative sandwich ELISA (R&D Systems, Abingdon, UK). 50 µl of Assay Diluent was added to wells coated with mouse monoclonal antibody against TNF-α. 200 µl of sample and standard was added and incubated for 2 hours at room temperature. After 4 washes, 200 µl of TNF-α Conjugate was then added to each well and incubated for 2 hours at room temperature. After further 4 washes, 200 µl of Substrate Solution was added to each well and incubated for 20 minutes in the dark at room temperature, following which 50 µl of Stop Solution was added. OD was determined using a microplate reader set at 450 nm and the concentration calculated from the standard curve.

3.4.6.17 Interleukin 6 (IL-6)

IL-6 was measured using solid phase sandwich ELISA (R&D Systems, Abingdon, UK). Capture Antibody was diluted in PBS to working concentration without carrier protein. 100 µl was added to each well, sealed and incubated overnight at room temperature. After 3 washes, 300 µl of Reagent Diluent was added to each well and incubated for 1 hour at room temperature. This followed by the addition of 100 µl of sample which is then incubated for 2 hours at room temperature. 100 µl of Detection Antibody is then added to each well and further incubated at room temperature for 2 hours. Following this, 100 µl of working dilution of Streptavidin-HRP was added to each well and incubated for 20 minutes. Each of the wells were washed 3 times in between each of the above steps. 100 µl of Substrate Solution was then added to each well and 50 µl of Stop Solution added following a further 20 minutes. OD of each well is determined using a microplate reader set to 540 nm and the concentration is calculated from the standard curve.

3.4.6.18 Glucose

Glucose was measured using enzymatic colorimetric method with glucose oxidase and 4-aminoantipyrine (GOD-PAP). The increase in absorbance at 510 nm was measured using Cobas Mira analyser (Horiba ABX Diagnostics, United Kingdom).

3.4.6.19 Insulin

Insulin was measured using a sandwich ELISA method (Mercodia AB, Uppsala, Sweden). Sample and standard were added to pre-coated micro-tire plate and peroxidase-conjugated secondary antibody was then added to create an antigen complex. After 1 hour of incubation followed by washing, Substrate Solution (Tretramethylbenzidine) was added following by the Stop Solution. OD was determined at 450 nm using a microplate reader and the concentration calculated from the standard curve.

3.4.6.20 Homeostatic model assessment of insulin resistance (HOMA-IR)

Insulin resistance was quantified using the HOMA-IR equation (11).

$$\text{HOMA-IR} = \frac{\text{Insulin (mU / l) x Glucose (mmol / l)}}{22.5}$$

3.4.6.21 Cystatin C

Serum cystatin C was measured using immunoturbidimetric assay. Sample was added to Cystatin Assay Buffer and Cystatin Antibody Reagent. The signal generated at 570 nm was measured after 10 and 15 minutes using Cobas Mira analyser (Horiba ABX Diagnostics, United Kingdom). The intra- and inter-assay CV were 2.6 and 4.4% respectively.

3.4.6.22 Bone profile

Total vitamin D (25-hydroxyvitamin D), phosphate, alkaline phosphatase (ALP), parathyroid hormone (PTH) and total calcium were measured using standard laboratory methods of the Department of Biochemistry, Salford Royal NHS Foundation Trust. Adjusted calcium was derived using the formula,

Adjusted calcium (mmol / l) = Total calcium (mmol / l) + 0.02 x [40 – Albumin (g / l)]

3.4.6.23 Sex hormones

Serum total testosterone, dihydrotestosterone, dehydroepiandrosterone sulphate and androstenendione levels were measured using liquid chromatography–tandem mass spectrometry in the validated clinical laboratory of Department of Biochemistry, Manchester University NHS Foundation Trust (12, 13). Sex hormone-binding globulin (SHBG), luteinising hormone and follicle-stimulating hormone levels were measured using electrochemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland) on Roche® automated analysers (E170 platform). Serum free testosterone levels were derived from patient's total testosterone, SHBG and albumin levels using the mass action equation described by Vermeulen (14). Low testosterone was defined as either, total testosterone level less than 8 nmol/L, or total testosterone level between 8 and 11 nmol/L with calculated free testosterone level less than 220 pmol/L(3).

3.4.7 Gluteal subcutaneous adipose tissue

3.4.7.1 Gluteal subcutaneous adipose tissue biopsy procedure

Gluteal subcutaneous fat biopsies were carried out at baseline and 6 months after bariatric surgery. The procedure was done in a sterile setting in the NIHR/Wellcome Trust Clinical Research Facility. Patients were asked to lie on their side and following strict sterile procedure, a small incision was made in the skin after pre-injection with local anaesthesia. The subcutaneous tissue was accessed, and 1-5 g of adipose tissue was collected. Any skin excision was sutured and dressed. Following this, written instructions about wound care and follow-up were given to the patient.

The tissue was immediately taken to the laboratory in a sealed container placed on ice. In the laboratory, the tissue was washed with saline to remove the remaining blood. Part of the sample was placed into RNAlater to allow for the preservation of messenger RNA. The remainder was divided and snap-frozen in dry ice. Samples were stored at -200 C.

3.4.7.2 Targeted gene expression in adipose tissue

RNeasy lipid tissue mini kit (Qiagen) was used for adipose tissue RNA extraction and reverse transcription was done with the QuantiTect reverse transcription kit (Qiagen). The cDNA concentration and purity were determined by a NanoDrop Lite spectrophotometer (ThermoFisher Scientific). Relative gene expression was determined using a LightCycler 480 machine (Roche) running LightCycler 480 SW 1.5.0 SP3 software. The assays used in Chapter 5 were Roche RealTime ready single assays (ABCA1, ABCG1, SCARB1 and TNF-α) relative to two control genes (ACTB and RN18S1). Genes were assayed in triplicate (reaction efficiencies were 100%) and 50ng of total cDNA was used per reaction for all genes except TNF-α for which required 100ng of total cDNA was used per reaction.

3.4.7.3 Immunohistochemistry

Adipose tissue immunohistochemistry was undertaken Dr Maria Jeziorska's lab. The allocated adipose tissue section was fixed using 4% paraformaldehyde in phosphate buffered saline for a 24-hour period and then processed into paraffin wax blocks.

3.4.7.4 Macrophage density

For assessment of macrophage density, consecutive tissue sections were immunostained for macrophage markers CD68 (KP1) (Dako, Glostrup, Denmark) and CD68 (PGM1) (Biocare Medical, Concord, California, USA), both at 1:100 dilution. This is followed by antigen retrieval and blocking of endogenous peroxidase and non-specific protein binding using Dako blocking solutions. Tissue sections were incubated with primary antibodies for 18 hours for antigen retrieval, then followed by anti-rabbit/anti-mouse EnVision-HRP (Dako, Glostrup, Denmark) for blocking of endogenous peroxidase and non-specific protein binding. Vector SG chromogen kit (Vector Laboratories, Burlingame, California, USA) was then used to demonstrate

the presence of macrophages. Colour images were captured with a Go-3 QImaging camera (QImaging Corporation, Vancouver, Canada) which is mounted on a Leitz Diaplan microscope (Leica, Wetzlar, Germany). Cells stained with macrophage markers were counted and expressed as cells/mm^2.

3.4.7.5. TNF-α immunostaining

For assessment of TNF-α, consecutive tissue sections were immunostained for TNF-α (Abcam, Cambridge, UK) at 1:100 dilution. Colour images were captured with a Go-3 QImaging camera (QImaging Corporation, Vancouver, Canada) mounted on a Leitz Diaplan microscope (Leica, Wetzlar, Germany). Quantitative analysis of immunostaining was undertaken using ImagePro version 6.2 image analysis program (MediaCybernetics United Kingdom, Marlow, UK) and the results expressed as a percentage of the area photographed.

3.4.7.6 Adipocyte size

Microphotographs were obtained on Zeiss Axio Imager M2 microscope with AxioCam camera. Adipocyte size was then quantified by free-hand tracing of the margins of 100 consecutive cells per case on the AxioVision Rel. 4.8 program and the adipocyte area calculated.

3.5 Statistical analysis

This section provides an overview on the statistical analysis undertaken in this study. Detailed descriptions of statistical analysis performed are further provided within individual results chapters.

Statistical analysis was performed using SPSS for Mac (Version 23.0, IBM SPSS Statistics, Armonk, New York, USA) and figures were produced using GraphPad Prism for Mac (Version 7.00, GraphPad Software, La Jolla California, USA). Continuous variables were assessed for normality using the Shapiro-Wilk test and visualisation of histograms and Q-Q plots. Results were presented as mean ± standard deviation (SD) for parametric and mean and interquartile range for non-parametric variables.

Comparisons between groups at baseline were made using the independent samples t-test for parametric variables and Mann Whitney U test for non-parametric variables. The chi-squared test was used for comparison of categorical variables. For comparisons before and after bariatric surgery, paired sample t-tests were used for parametric variables, Wilcoxon matched-pairs signed rank test for non-parametric variables, and the McNemar test for categorical variables. For comparisons between more than two time points, the one-way ANOVA was used for parametric variables and Friedman's two-way analysis of variance by ranks was used for non-parametric variables.

Correlation analysis was done using Pearson's test for parametric and Spearman's test for non-parametric variables.

Automated Bonferroni and Dunn's corrections were incorporated for multiple testing (using built-in features in SPSS and GraphPad Prism). Multiple regression and binary logistic regression analysis were undertaken in SPSS and predictive factors were either chosen from pre-specified hypothesis or prior univariate analysis.

No adjustments were made for missing data. The level of statistical significance was set at less than 0.05 for all analyses.

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Chapter 4: Bariatric surgery as a model to explore the basis and consequences of the Reaven hypothesis: Small, dense low-density lipoprotein and interleuklin-6

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Bariatric surgery as a model to explore the basis and consequences of the Reaven hypothesis: Small, dense low-density lipoprotein and interleukin-6

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Abstract

Background: Reaven originally described the clustering of insulin resistance/hyperinsulinaemia, obesity (particularly visceral), altered cytokine levels, glucose intolerance, hypertriglyceridaemia and low high-density lipoprotein cholesterol. Subsequently, a potentially highly atherogenic small, dense low-density lipoprotein was also reported. We have studied the effect of bariatric surgery on this and other risk factors for atherosclerosis.

Methods: Forty patients (20 with type 2 diabetes mellitus) undergoing bariatric surgery were studied before and 1 year after bariatric surgery.

Results: Twelve months after bariatric surgery, median body mass index had decreased from 49.5 to 36.5 kg/m^2, fasting insulin from 21.3 to 7.8 mU/L and insulin resistance (homeostatic model assessment of insulin resistance) from 5.9 to 1.8 (all $p < 0.001$). Thirteen out of 20 patients had remission from type 2 diabetes mellitus. Highly sensitive C-reactive protein, interleukin-6, fasting triglycerides ($p < 0.001$) and small, dense low-density lipoprotein ($p < 0.001$) decreased, while high-density lipoprotein cholesterol increased ($p < 0.001$) significantly, irrespective of having type 2 diabetes mellitus and/or being treated with statin therapy before surgery.

Conclusion: The association between marked weight loss and change in insulin resistance and hyperinsulinaemia with the change in small, dense low-density lipoprotein and interleukin-6 warrants further investigation. Bariatric surgery provides a model for investigating the mechanisms linking insulin resistance/hyperinsulinaemia to atherosclerosis.

Keywords

Insulin resistance, Reaven's hypothesis, bariatric surgery, obesity, metabolic syndrome, triglycerides

Introduction

The advent of reliable plasma insulin assays in the 1960s provided the opportunity for Gerald Reaven et al.[1] to discover the association between the insulin response to carbohydrate feeding and serum triglyceride levels. Higher insulin responses were associated with higher triglyceride levels.[1] Reaven and others went on to report that a constellation of other abnormalities was also associated with an exaggerated insulin response in addition to hypertriglyceridaemia, including type 2 diabetes mellitus (T2DM) or a predisposition to it, high-density lipoprotein cholesterol (HDL-C), non-alcoholic steatohepatitis, hypertension, hyperuricaemia and raised plasminogen activator inhibitor-1 (PAI-1), fibrinogen and highly sensitive

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C-reactive protein (hs-CRP).[2–5] This, he termed 'Syndrome X',[2] although it is now more widely known as the metabolic syndrome, particularly when associated with obesity. Initially, Reaven's hypothesis stated that increased insulin levels were the cause of the hypertriglyceridaemia, because insulin was at that time believed to stimulate hepatic very low-density lipoprotein (VLDL) secretion.[1] However, later experiments with adult hepatocytes maintained in tissue culture showed that the primary effect of insulin on hepatic VLDL secretion was inhibitory.[6–8] Thus, the hypertriglyceridaemia was due to insulin resistance, rather than hyperinsulinaemia, as previously proposed by Himsworth[9] many years previously. Reaven thus modified his hypothesis and extended it to state that both muscle and the liver must be resistant to the action of insulin, at least in relation to their diminished capacity for the uptake of glucose, and therefore, the increased insulin levels were a response to overcome the insulin resistance. That hepatic insulin resistance could also explain the hypertriglyceridaemia of metabolic syndrome and T2DM, was later confirmed in human studies of VLDL kinetics.[10–12] Throughout his life Reaven continued to argue, however, that it was impossible using available techniques and models to separate the effects of insulin resistance from the effects of too much insulin in humans.[13] It is known that insulin resistance due to inherited insulin receptor defects results in reduced glucose uptake and hyperinsulinaemia, but does not lead to hypertriglyceridaemia or hepatic steatosis.[14] However, after its uptake by functioning receptors, insulin regulates multiple intracellular pathways through several signalling mechanisms.[15] It therefore remains entirely possible that some of these are resistant to insulin, whereas, as Reaven originally postulated, others are over-stimulated by the hyperinsulinaemia which develops to maintain euglycaemia. When the increased delivery of insulin is inadequate to overcome insulin resistance to glucose uptake, T2DM develops, but the insulin levels are much higher than in healthy, non-obese, people without diabetes.[16]

The provision of evidence that hyperinsulinaemia/insulin resistance is causal for hypertriglyceridaemia and the other components of the metabolic syndrome was, until the advent of bariatric surgery, hampered by the lack of a means of dramatically reversing it. Drugs which decrease insulin resistance tend to have multiple other actions and the effect of weight reduction through dietary restriction can only be studied when a large proportion of failures are excluded. Bariatric surgery provides a means of substantially and consistently reversing hyperinsulinaemia/insulin resistance. While the substantial decrease in adiposity may explain this reversal of hyperinsulinaemia/insulin resistance, additional mechanisms such as changes in gut hormone profiles due to intestinal bypass may contribute. We, and others, have previously reported a decrease in elevated levels of inflammatory cytokines and an increase in adiponectin after bariatric surgery.[17–20] These cytokines emanate from adipose tissue and those from visceral adipose tissue, particularly interleukin-6 (IL-6), arrive at the liver via the portal circulation,[21] and are believed to be responsible for hepatic resistance to insulin-mediated glucose uptake.[22]

Quite why the metabolic syndrome is associated with an increased risk of atherosclerotic cardiovascular disease (CVD) has never been fully explained. Increases in low-density lipoprotein cholesterol (LDL-C) and in its major protein moiety, apolipoprotein B (ApoB), which are definitely causal, are not a feature of metabolic syndrome.[2,23] Moderate hypertriglyceridaemia, typical of the metabolic syndrome has proved controversial as a cause of CVD,[24] and the role of high-density lipoprotein (HDL) in atherogenesis is currently being re-evaluated.[25] We have previously made a preliminary report of a decrease in small, dense low-density lipoprotein (SD-LDL) following bariatric surgery.[18] SD-LDL is increased in hypertriglyceridaemia [26–28] and is particularly susceptible to atherogenic modifications, such as oxidation and glycation.[29,30] Inflammatory cytokines associated with atherothrombosis may also make a major contribution to CVD in metabolic syndrome.[31] In the present study, we have undertaken a comprehensive assessment of the effect of bariatric surgery on insulin secretion and insulin resistance in relation to the change in SD-LDL and IL-6, an upstream regulator of C-reactive protein (CRP),[4] as there is emerging evidence from Mendelian randomisation studies that it has a longer term association with CVD than hs-CRP.[32,33] We have sought to establish the basis for the change in insulin secretion and insulin resistance after bariatric surgery, to further understand the basis of the Reaven hypothesis.

Methods

Study design and patient recruitment

This study was a prospective observational cohort study. Forty patients (8 men and 32 women) with obesity were recruited from the pre-bariatric surgery clinic at Salford Royal Hospital, a Tier 4 specialist weight management service in the North West of England. They all underwent Roux-en-Y laparoscopic gastric bypass surgery.

Prior ethical approval was sought and granted by the Central Manchester Research and Ethics Committee. All patients were given detailed information about the study and each person provided informed consent before taking part in the study.

Patient assessments

Patients were asked to attend a morning appointment (between 09:00 and 10:30 h) having fasted from 22:00 h at baseline and then 6 and 12 months after bariatric surgery.

At each visit, a detailed medical history including medication used was assessed. Each participant underwent measurement of their weight and height and body mass index (BMI) was calculated. Blood pressure was measured after resting in a seated position for 15 min, using an Omron HEM 705-CP semiautomatic oscillometric recorder. Fasting venous blood was collected at each visit. Metabolic syndrome was defined using the current revision from the National Cholesterol Education Programme Adult Treatment Panel III.[23]

Complete remission from type 2 diabetes was determined 12 months post-operatively with glycated haemoglobin (HbA1c) below 6.0% (42 mmol/mol) and no active pharmacological therapy, as per the American Diabetes Association consensus statement.[34]

Laboratory procedures and analyses

HbA1c and fasting glucose were assessed in the biochemistry laboratory at Manchester University Hospitals NHS Foundation Trust using routine methods. The remaining samples were processed in the Cardiovascular Research Lab at the University of Manchester. Laboratory procedures and measurements were carried out according to our previously described protocol.[18] Serum and ethylenediaminetetraacetic acid (EDTA)-plasma were isolated by centrifugation at 2000 × *g* for 15 min at 4°C within 2 h of blood collection. Aliquots for biochemical analysis were frozen at −80°C.

Total cholesterol was measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase method and triglycerides by the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase method. HDL-C was measured using a second-generation homogeneous direct method (Roche Diagnostics, Burgess Hill, UK). LDL-C was estimated using the Friedewald formula.[35] ApoB was measured using immunoturbidimetric assays (ABX Diagnostics, Shefford, UK). All these tests were performed on a Cobas Mira analyser (Horiba ABX Diagnostics, Nottingham, UK).

Small, dense low-density lipoprotein apolipoprotein B (SD-LDL ApoB; LDL particles of density > 1.044 g/mL) was isolated from plasma and ultracentrifuged at 100,000 r/min (435,680 × *g*) for 5 h at 4°C using a Beckman Optima TLX bench top ultracentrifuge fitted with TLA 120.2 fixed angle rotor (Beckman Coulter UK).[36] ApoB in SD-LDL was then determined using an immunoturbidimetric assay (ABX Diagnostics). SD-LDL is thus expressed in terms of the plasma concentration of its ApoB component.

An in-house, antibody sandwich enzyme-linked immunosorbent assay (ELISA) technique using anti-human CRP antibody, calibrators and controls from Abcam (Cambridge, UK) was used to measure hs-CRP. IL-6 was measured by ELISA using kits from R&D Systems (Abingdon, UK). The upper limit (95th percentile) for IL-6 in plasma was 3.1 pg/mL.[37] Plasma insulin was measured with Mercodia ELISA kits from Diagenics Ltd. (Milton Keynes, UK). Homeostatic model assessment of insulin resistance (HOMA-IR) was used to assess insulin resistance,[38] using the formula

$$\text{HOMA-IR} = \left[\text{insulin}(\text{mU/L}) \times \text{glucose}(\text{mmol/L})\right]/22.5$$

The laboratories participated in the UK National External Quality Assessment Service (UKNEQAS, Birmingham, UK) for quality control of general blood chemistry.

Statistical analysis

SPSS for Mac (Version 23.0; IBM SPSS Statistics, IBM Corp., Armonk, NY) and GraphPad Prism (Version 7.00; GraphPad Software, La Jolla, CA, USA) were used for analysis of data. Tests for normality were done using the Shapiro–Wilk test, visualisation of histograms and Q-Q plots. When more than two time points were being compared, one-way ANOVA was used for parametric data and Friedman's two-way analysis of variance by Ranks was used for non-parametric data. Specific post hoc pairwise comparisons were done using the Bonferroni correction in SPSS. The McNemar test was used to compare paired categorical variables. The percentage change in variables was determined as the absolute difference between measurements 12 months after surgery and baseline divided by the baseline value (and multiplied by 100). Correlation analysis was done using Pearson's test for parametric and Spearman's test for non-parametric data. A p-value of <0.05 was considered to be statistically significant.

Results

Clinical characteristics

The baseline and post-operative clinical measures are given in Table 1. The mean age of participants was 48 years. BMI, waist circumference and systolic blood pressure were reduced significantly ($p < 0.05$), with no significant change in diastolic blood pressure ($p = 0.15$). Of the 40 patients, 20 had T2DM pre-operatively and remitted completely in 13 out of 20 (65%) patients 12 months after surgery ($p < 0.001$). There was a significant reduction in the number of participants meeting the diagnostic criteria for the metabolic syndrome ($p < 0.001$) and there was a trend towards reduction in the use of lipid-lowering drugs ($p = 0.06$) after bariatric surgery.

Laboratory measurements

Results of the laboratory measurements are shown in Table 2 and Figure 1. In the entire cohort, there were significant ($p < 0.05$) reductions in the triglycerides, HDL-C, SD-LDL ApoB, hs-CRP, IL-6, HbA1c, glucose, insulin

Table 1. Clinical parameters before and after bariatric surgery.

	Baseline	6 months	12 months	p
Age at time of surgery (years)	48 (8)			
Female (%)	32 (80)			
Diabetes presence (%)	50.0		17.5	**<0.001**
Lipid-lowering drugs (%)	57.5		45.0	0.063
Metabolic syndrome (%)	72.5		22.5	**<0.001**
Body mass index (kg/m^2)	49.5 (45.0–57.0)	36.5 (32.8–44.0)†††	33.0 (30.0–38.8)§§§,***	**<0.001**
Waist circumference (cm)	137 (128–150)	112 (106–125)†††	104 (95.0–115)§§,***	**<0.001**
Systolic blood pressure (mmHg)	131 (120–145)	127 (110–140)	119 (109–134)*	**0.025**
Diastolic blood pressure (mmHg)	75.0 (13.0)	75.0 (13.0)	70.0 (11.0)	0.150
Excess body mass index loss (%)			65.9 (18.0)	

Clinical parameters at baseline, 6 and 12 months after bariatric surgery. Data represented as mean (SD) or median (IQR). Percentages in categorical variables represent proportion of participants with type 2 diabetes, using lipid-lowering therapy or fulfilling the diagnostic criteria for the metabolic syndrome. Remission of type 2 diabetes determined with a glycated haemoglobin below 42 mmol/mol (6.0%) and no active pharmacological therapy as per the American Diabetes Association consensus statement. Metabolic syndrome defined using the current National Cholesterol Education programme Adult Treatment Panel III.
Bold values shows statistically significant results.
Baseline compared to 6 months: *$p<0.05$; **$p<0.01$; ***$p<0.001$.
Baseline compared to 12 months: $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$.
6 months compared to 12 months: $^{\S}p<0.05$; $^{\S\S}p<0.01$; $^{\S\S\S}p<0.001$.

and HOMA-IR, 12 months post-operatively with intermediate values at 6 months. Total ApoB levels were significantly reduced only at 12 months and total cholesterol and LDL-C did not change significantly.

Subgroup analysis of the patients with and without diabetes showed similar results, except patients without diabetes had a significant reduction ($p=0.01$) in LDL-C compared to those with diabetes. The significant improvements in triglycerides, HDL-C, SD-LDL ApoB, hs-CRP, IL-6, HbA1c, glucose, insulin and HOMA-IR were seen both in patients using statins (n=23) and those not on statins (n=17). Those using statins had higher serum total ApoB, reflecting a more severe dyslipidaemia phenotype. Patients not on statin therapy showed a greater reduction in total ApoB, but this was not statistically significant ($p=0.12$) (Supplementary Table 1).

Relationships between fasting insulin, HOMA-IR and other metabolic variables

Correlations of values at baseline and 12 months post-operatively

The relationships at baseline between fasting insulin and BMI are illustrated in Figure 2(a) and with HOMA-IR and BMI in Figure 2(c). The association between post-operative fasting insulin and BMI is shown in Figure 2(b) and for HOMA-IR and BMI in Figure 2(d).

Both pre-operatively and 1 year post-operatively, fasting insulin levels correlated significantly with HDL-C levels (r=–0.37; $p=0.02$ and r=–0.40; $p=0.01$, respectively). HOMA-IR measurements showed a significant association with triglycerides (r=0.34; $p=0.03$) and HDL-C (r=–0.39; $p=0.01$) at baseline and at 12 months (triglycerides r=0.36; $p=0.02$, HDL-C r=–0.42; $p=0.007$). Triglycerides were not significantly correlated with insulin (r=0.22; $p=0.18$) at baseline, but were weakly correlated at 12 months post-operatively (r=0.31; $p=0.05$). Pre-operatively, IL-6 correlated with BMI (r=0.43; $p=0.009$), but not with insulin levels or HOMA-IR. The relationship with BMI was weaker post-operatively (r=0.31; $p=0.07$) possibly because of the loss of visceral fat. IL-6 correlated with hs-CRP post-operatively (r=0.42; $p=0.007$).

Association of change in hyperinsulinaemia and insulin resistance

The percentage change (Δ) in fasting insulin levels between the pre- and post-operative state was related to Δtriglycerides (r=0.36; $p=0.03$), Δhs-CRP (r=0.42; $p=0.01$), ΔIL-6 (r=0.41; $p=0.02$) and ΔBMI (r=0.43; $p=0.007$). There were also significant correlations between ΔHOMA-IR with Δtriglycerides (r=0.33; $p=0.04$), Δhs-CRP (r=0.37; $p=0.02$) and ΔIL-6 (r=0.37; $p=0.03$).

Discussion

This study shows that a marked reduction in hyperinsulinaemia/insulin resistance in obese people after bariatric surgery ameliorates not only raised triglycerides, hs-CRP and low HDL-C, features of the Reaven syndrome,[2] but also SD-LDL and IL-6, irrespective of the presence of T2DM or statin therapy.

SD-LDL concentration increases with triglyceride levels.[26] It is a cholesterol-depleted low-density lipoprotein

Table 2. Laboratory values before and after bariatric surgery.

	All patients (n = 40)				Diabetes (n = 20)				Non-diabetes (n = 20)			
	Baseline	6 months	12 months	*p*	Baseline	6 months	12 Months	*p*	Baseline	6 months	12 months	*p*
TC (mmol/L)	4.74 (3.88–5.34)	4.56 (3.86–5.63)	4.53 (3.99–5.20)	0.588	4.06 (3.75–5.16)	4.43 (3.86–5.16)	4.23 (3.81–5.03)	0.963	5.00 (4.41–5.86)	5.17 (3.90–5.79)	4.64 (3.38–4.64)	0.359
Triglycerides (mmol/L)	1.68 (1.20–2.25)	1.45 (1.02–1.67)	1.10§§,*** (1.01–1.35)	**<0.001**	1.88 (1.27–2.64)	1.49 (1.30–1.70)	1.16*** (1.01–1.43)	**<0.001**	1.41 (1.09–2.00)	1.27 (1.01–1.61)	1.05* (0.87–1.35)	**0.010**
HDL-C (mmol/L)	1.18 (1.03–1.34)	1.18 (1.05–1.51)	1.40§§,*** (1.28–1.74)	**<0.001**	1.11 (1.01–1.39)	1.17 (1.09–1.51)	1.40§§,** (1.24–1.73)	**0.001**	1.23 (1.05–1.32)	1.19 (1.01–1.51)	1.46§,** (1.30–1.76)	**0.002**
LDL-C (mmol/L)	2.53 (1.95–3.21)	2.79 (1.89–3.87)	2.38 (1.96–3.22)	0.122	2.03 (1.75-2.62)	2.29 (1.85–3.35)	2.15 (1.78–2.73)	0.086	3.10 (2.36–3.92)	3.00 (2.48–3.88)	2.75** (2.03–3.30)	**0.011**
Total ApoB (g/L)	0.94 (0.79–1.11)	0.96 (0.76–1.23)	0.86 (0.74–1.06)	**0.032**	0.86 (0.78–1.00)	0.94 (0.77–1.25)	0.83 (0.69–1.04)	0.120	0.96 (0.78–1.19)	0.96 (0.75–1.23)	0.87 (0.79–1.10)	0.099
SD-LDL ApoB (mg/dL)	22.1 (16.6–30.5)	11.5††† (8.90–18.7)	10.2*** (6.8–15.6)	**<0.001**	21.6 (17.8–28.9)	11.7††† (8.44–20.5)	10.2*** (6.87–17.2)	**<0.001**	22.6 (11.3–30.6)	11.2 (9.13–18.4)	10.1*** (6.75–14.1)	**0.001**
hs-CRP (mg/L)	6.20 (4.11–10.3)	2.66††† (1.10–5.00)	1.07§,*** (0.50–2.92)	**<0.001**	5.78 (3.85–9.29)	2.6†† (0.7–3.96)	1.21*** (0.65–2.52)	**<0.001**	6.25 (5.18–11.3)	3.69 (1.25–5.40)	1.00§§,*** (0.47–3.06)	**<0.001**
IL-6 (pg/mL)	2.98 (1.44–5.61)	1.55††† (0.38–4.13)	1.26*** (0.17–3.06)	**<0.001**	3.99 (2.39–7.86)	1.39††† (0.78–3.43)	1.26*** (0.41–4.50)	**<0.001**	2.72 (1.09–3.60)	1.80 (0.06–5.09)	1.01** (0.06–2.87)	**0.007**
HbA1c (mmol/mol)	45 (41–56)	39††† (34–41)	36§,*** (33–40)	**<0.001**	56 (48–75)	40†† (35–46)	37*** (34–41)	**<0.001**	42 (38–44)	37 (33–40)	35* (32–37)	**<0.001**
Glucose (mmol/L)	6.03 (5.24–7.00)	5.29†† (4.83–6.01)	4.89*** (4.67–5.82)	**<0.001**	6.98 (5.24–12.5)	5.97 (4.90–8.59)	5.66** (4.67–6.96)	**0.004**	5.76 (5.14–6.14)	5.08† (4.70–5.62)	4.80*** (4.65–5.10)	**<0.001**
Insulin (mU/L)	21.3 (14.5–37.8)	9.04††† (6.73–13.9)	7.75*** (5.60–11.7)	**<0.001**	22.9 (13.8–55.9)	9.60† (6.58–16.5)	8.90*** (5.60–16.4)	**<0.001**	19.9 (15.3–26.6)	8.58†† (6.77–13.7)	7.34*** (5.22–10.8)	**<0.001**
HOMA-IR	5.89 (4.17–11.1)	2.56††† (1.54–4.34)	1.76*** (1.22–2.65)	**<0.001**	8.24 (5.53–13.7)	2.68† (1.80–4.93)	2.39*** (1.22–4.13)	**<0.001**	5.47 (3.48–6.81)	1.92† (1.52–3.18)	1.58*** (1.13–2.31)	**<0.001**

TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; ApoB: apolipoprotein B; SD-LDL ApoB: small, dense low-density lipoprotein apolipoprotein B; hs-CRP: highly sensitive C-reactive protein; IL-6: interleukin-6; HbA1c: glycated haemoglobin; HOMA-IR: homeostatic model assessment of insulin resistance.
Laboratory variables at baseline, 6 and 12 months after bariatric surgery in the entire cohort, patients with diabetes and patients without diabetes. Values represented as median (interquartile range).
Bold values shows statistically significant results.
Baseline compared to 6 months: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Baseline compared to 12 months: †$p < 0.05$; ††$p < 0.01$; †††$p < 0.001$.
6 months compared to 12 months: §$p < 0.05$; §§$p < 0.01$; §§§$p < 0.001$.

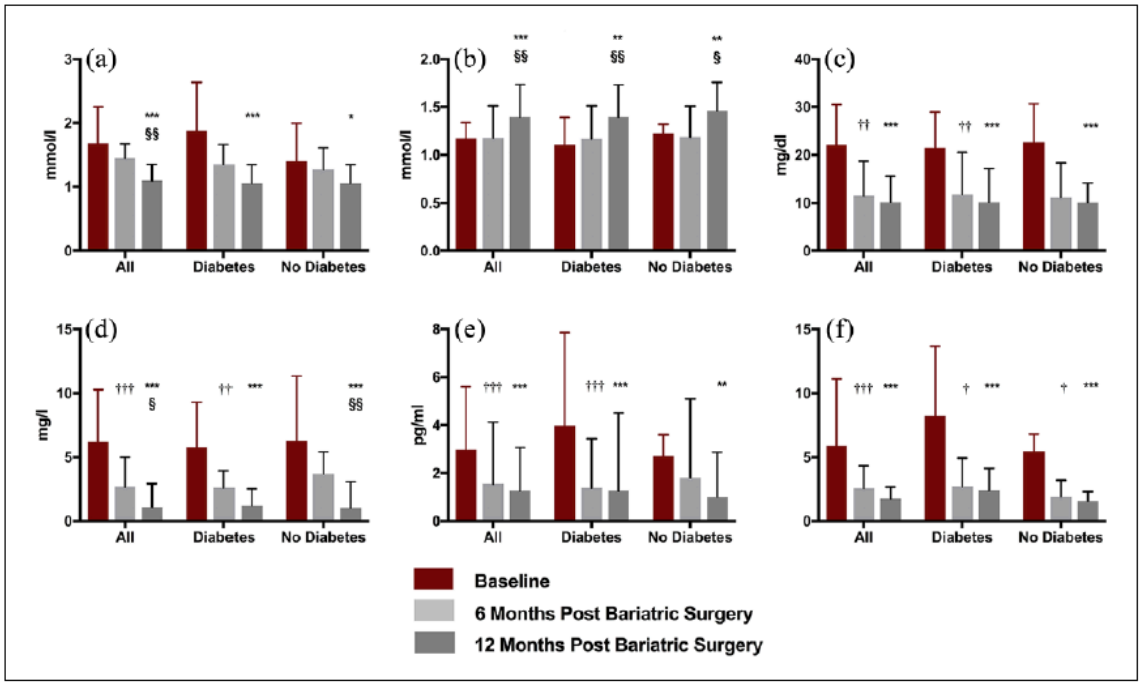

Figure 1. The responses to bariatric surgery in the whole 40 patients and in those with and without type 2 diabetes separately before and at 6 and 12 months post-operatively in (a) fasting triglycerides, (b) high-density lipoprotein cholesterol (HDL-C), (c) small, dense low-density lipoprotein apolipoprotein B (SD-LDL ApoB), (d) highly sensitive C-reactive protein (hs-CRP), (e) interleukin-6 (IL-6) and (f) insulin resistance (HOMA-IR). Bars are median + 75th percentile.
Baseline compared to 6 months: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.
Baseline compared to 12 months: $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$.
6 months compared to 12 months: $^{\S}p<0.05$; $^{\S\S}p<0.01$; $^{\S\S\S}p<0.001$.

(LDL), the presence of which is not evident from measurement of LDL-C. It contributes to total serum ApoB and is the cause of hyperapobetalipoproteinaemia,[39] but the majority of ApoB-containing lipoproteins are of lower density so that even when total ApoB is measured its presence may not be obvious. It is likely that it is the cause of the association between hypertriglyceridaemia and CVD.[24,26,27] SD-LDL is more susceptible both to oxidative and glycative modification than less dense LDL species.[29,30] Both oxidatively modified and glycated LDL, unlike more buoyant, unmodified LDL, are rapidly taken up via scavenger receptors on macrophages in tissue culture to become foam cells similar to those in atheromatous lesions.[40] In our series of patients, the SD-LDL concentration of 22.1 mg/dL at baseline declined to 10.2 mg/dL 12 months after bariatric surgery, restoring its levels close to the median value in a healthy population (14 mg/dL for men and 9 mg/dL for women).[41]

A raised level of hs-CRP is recognised as a feature of metabolic syndrome[2] and is closely associated to the risk of developing CVD.[4] However, evidence that hs-CRP is causally related to CVD has not been forthcoming.[4] On the other hand IL-6, a major regulator of hepatic CRP secretion, unlike CRP, has been found in Mendelian randomisation studies to be linked to atherosclerosis.[4,32,33] Many adipokines are released both from peripheral and visceral adipose tissue, which have the potential to contribute to hepatic insulin resistance and secretion of CRP, but those released from visceral fat may have a special place in the genesis of the metabolic syndrome, because they arrive at the liver through the portal vein and may do so at higher concentration than those arriving by the hepatic artery after dilution in the systemic circulation. IL-6 was found in much higher concentration in portal blood than in systemic arterial blood by Fontana et al.[21] in patients undergoing gastric bypass surgery for obesity. Tumour necrosis factor-α, resistin, macrophage chemoattractant protein-1 and adiponectin concentrations were similar in the portal vein and radial artery. Portal vein IL-6 concentration also correlated directly with systemic CRP. The decrease in IL-6 after bariatric surgery, suggests that it could be associated with the decrease in hs-CRP and other features of hepatic insulin resistance. Recently, reduction in IL-6 levels similar to that reported here, but achieved by administration of a monoclonal antibody to interleukin-1β, was reported to be associated with decreased atherosclerotic CVD incidence.[31]

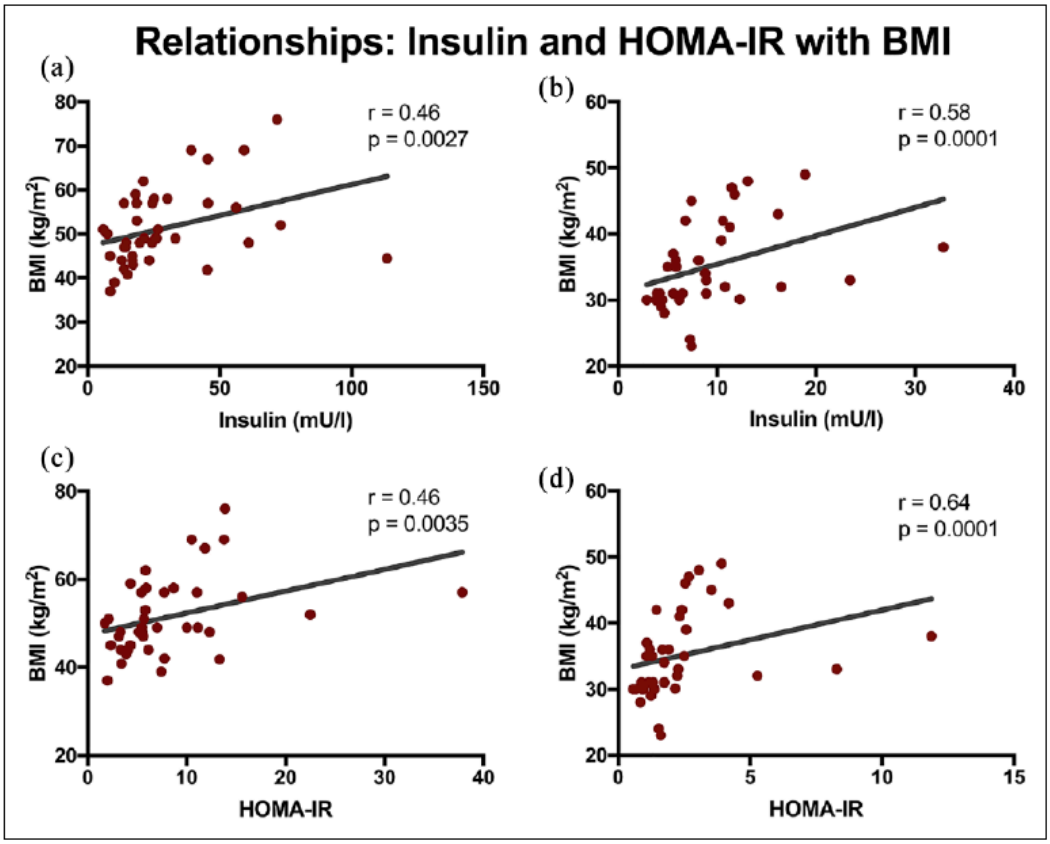

Figure 2. Fasting insulin and homeostatic model assessment of insulin resistance (HOMA-IR) as a function of body mass index (BMI) at baseline [panels (a) and (c)] and 12 months after bariatric surgery [panels (b) and (d)].

The findings of this and earlier reports in which the effects of the decrease in insulin resistance accompanying weight loss on components of the metabolic syndrome have been investigated,[17–20] provide powerful support for Reaven's hypothesis. Bariatric surgery which induces the most dramatic decreases in hyperinsulinaemia/insulin resistance is a model which could yield an even greater understanding of, for example, the mechanism by which atheroma risk is increased. Our finding of a decrease in SD-LDL should lead to exploration of the effects of weight loss due to bariatric surgery on modified, potentially highly atherogenic LDL subspecies, such as oxidised and glycated LDL. Furthermore, it could lead to some resolution of the conflict which exists between which components of the metabolic (Reaven) syndrome are due to resistance to insulin (too little insulin action) and which are due to hyperinsulinaemia itself (too much insulin action). In recent years, it has often been forgotten that, because insulin regulates several intracellular signalling pathways controlling a variety of processes, while its effects will be deficient in pathways resistant to it, in others the high levels of insulin produced to attempt to maintain euglycaemia may hyperstimulate non-resistant pathways. An example might be the regulation of sex hormone–binding globulin (SHBG), which is decreased in insulin resistance, leading to increased free androgen levels in both men and women.[42,43] This at least partly explains the androgenisation of insulin resistant women and thus their male pattern (visceral; central) obesity and hirsutism. Despite the association of insulin resistance with decreased SHBG, however, tissue culture experiments with human hepatocytes reveal insulin to have an inhibitory action on SHBG production.[44,45] Thus, unlike the VLDL production pathway where insulin resistance decreases the inhibitory effect of insulin despite the increase in its concentration in response to resistance to glucose uptake, the pathway for the production of SHBG must escape resistance to the action of insulin and be inhibited by hyperinsulinaemia. The model of bariatric surgery may provide opportunities for futher study of this mechanism as it may for other phenomena associated with the hyperinsulinaemia of insulin resistance, such as the pathways linking metabolic syndrome to hyperuricaemia.[46]

It has been difficult to distinguish components of the metabolic syndrome due to resistance to insulin action

and those hyperstimulated by the ensuing increase in insulin secretion to maintain euglycaemia as first discussed in Kim and Reaven.[13] We found that the strength of the associations between change in components of the metabolic syndrome, such as triglycerides and HDL-C, and change in fasting insulin and HOMA-IR were similar with perhaps the suggestion that triglyceride concentration was more closely related to insulin resistance and HDL-C to fasting insulin levels. It has been suggested that the insulin clamp technique would be a better means of assessing insulin resistance rather than HOMA-IR, which relies on the ratio between fasting insulin and glucose.[38] However, this argument is less persuasive when it is considered that in insulin clamping, the insulin is administered into the systemic rather than the portal circulation into which it is secreted physiologically.[47] This means that physiologically the liver is subject to much higher levels than peripheral tissues and the insulin clamp is thus measuring insulin resistance to glucose uptake in tissues, such as skeletal muscle, while hepatic uptake is relatively unaffected by insulin arriving by the hepatic artery. Presumably, HOMA-IR, however, represents the contribution of both hepatic and peripheral glucose disposal. At the time of bariatric surgery, direct measurement of hormones and metabolites in portal venous blood can be undertaken,[21] but repetition of this after weight loss is not possible, at least in human models. Nonetheless, discovering which processes are resistant to insulin and which are over-stimulated by the accompanying hyperinsulinaemia could be important therapeutically.

We conclude that bariatric surgery provides an excellent model to dissect Reaven's hypothesis, to discover the mechanisms by which its components, such as raised SD-LDL and IL-6, cause atherosclerosis. It also raises questions about the respective roles of insulin resistance and of hyperinsulinaemia on different components of the metabolic syndrome.

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Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: H.S. and P.N.D. have served as consultants to pharmaceutical companies marketing lipid-lowering drugs and have received travel expenses, payment for speaking at meetings and funding for research from some of these companies. None of the other authors have any relevant conflicts of interest to declare.

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Supplemental material

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Supplemental Material for publication

Supplementary Table 1: Laboratory Values before and after Bariatric Surgery based on Statin Use

	All Patients (n=40)				Statin (n=23)				Non-Statin (n=17)			
	Baseline	**6 Months**	**12 Months**	**p**	**Baseline**	**6 Months**	**12 Months**	**p**	**Baseline**	**6 Months**	**12 Months**	**p**
TC mmol/l	4.74 (3.88-5.34)	4.56 (3.86-5.63)	4.53 (3.99-5.20)	0.588	4.42 (3.84-5.23)	4.44 (3.84-5.69)	4.49 (3.78-5.21)	0.878	4.97 (4.24-5.60)	4.94 (3.87-5.63)	4.56 (4.14-5.28)	0.343
Triglycerides mmol/l	1.68 (1.20-2.25)	1.45 (1.02-1.67)	1.10§§*** (1.01-1.35)	**<0.001**	1.78 (1.20-2.53)	1.49 (1.28-1.70)	1.27§*** (1.05-1.56)	**<0.001**	1.43 (1.05-2.22)	1.13 (0.86-1.60)	1.03** (0.80-1.16)	**0.003**
HDL-C mmol/l	1.18 (1.03-1.34)	1.18 (1.05-1.51)	1.40§§*** (1.28-1.74)	**<0.001**	1.18 (1.01-1.41)	1.17 (1.01-1.50)	1.40§* (1.23-1.70)	**0.001**	1.17 (1.03-1.32)	1.20 (1.03-1.55)	1.47§§** (1.31-1.77)	**0.002**
LDL-C mmol/l	2.53 (1.95-3.21)	2.79 (1.89-3.87)	2.38 (1.96-3.22)	0.122	2.08 (1.82-2.91)	2.55 (1.74-3.59)	2.19 (1.75-3.30)	0.309	3.04 (2.38-3.65)	2.94 (2.04-3.87)	2.67 (2.11-3.20)	0.249
Total ApoB g/l	0.94 (0.79-1.11)	0.96 (0.76-1.23)	0.86 (0.74-1.06)	**0.032**	0.90 (0.78-1.08)	0.99 (0.74-1.23)	0.84 (0.73-1.13)	0.156	0.95 (0.80-1.12)	0.90 (0.75-1.23)	0.87 (0.76-0.97)	0.119
sdLDL ApoB mg/dl	22.1 (16.6-30.5)	11.5††† (8.90-18.7)	10.2*** (6.8-15.6)	**<0.001**	26.0 (17.5-31.5)	10.8† (6.96-13.7)	11.8*** (7.51-15.4)	**<0.001**	18.8 (12.6-25.1)	11.7 (8.78-22.9)	8.78§*** (6.58-16.1)	**0.001**
hsCRP mg/l	6.20 (4.11-10.3)	2.66††† (1.10-5.00)	1.07§*** (0.50-2.92)	**<0.001**	5.56 (4.21-9.32)	2.60†† (1.34-4.74)	1.66*** (0.85-3.16)	**<0.001**	6.52 (4.01-11.1)	2.66 (0.90-6.67)	0.81§§*** (0.36-2.71)	**<0.001**
IL-6 pg/ml	2.98 (1.44-5.61)	1.55††† (0.38-4.13)	1.26*** (0.17-3.06)	**<0.001**	3.43 (1.62-6.56)	1.19††† (0.00-3.40)	1.26*** (0.37-3.75)	**<0.001**	2.88 (1.09-3.81)	2.52 (0.89-5.09)	1.21** (0.06-2.59)	**0.007**
HbA1c mmol/mol	45 (41-56)	39††† (34-41)	36§*** (33-40)	**<0.001**	49 (44-70)	40†† (38-44)	37*** (35-41)	**<0.001**	42 (38-44)	34 (32-37)	34*** (32-36)	**<0.001**
Glucose mmol/l	6.03 (5.24-7.00)	5.29†† (4.83-6.01)	4.89*** (4.67-5.82)	**<0.001**	6.28 (5.27-8.01)	5.74 (4.87-7.43)	5.42*** (4.68-6.69)	**0.004**	5.80 (5.10-6.31)	5.04 (4.71-5.56)	4.77** (4.49-4.95)	**<0.001**
Insulin mU/l	21.3 (14.5-37.8)	9.04††† (6.73-13.9)	7.75*** (5.60-11.7)	**<0.001**	21.1 (13.8-45.4)	10.4† (7.56-13.9)	8.90*** (5.80-16.2)	**<0.001**	23.4 (15.8-36.3)	7.79†† (5.92-14.8)	7.29*** (4.73-10.1)	**<0.001**
HOMA-IR	5.89 (4.17-11.1)	2.56††† (1.54-4.34)	1.76*** (1.22-2.65)	**<0.001**	7.46 (5.48-12.3)	2.72 (1.80-4.70)	2.41 (1.32-3.92)	**<0.001**	5.46 (3.62-10.3)	1.77†† (1.23-3.40)	1.54*** (1.02-2.04)	**<0.001**

Supplementary Table 1. Laboratory variables at baseline, 6 and 12 months after bariatric surgery in the entire cohort, patients using statins and patients not using statins. Values represented as median (interquartile range). TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; ApoB: apolipoprotein B; sdLDL apoB: small dense low-density lipoprotein fraction of apolipoprotein B; hsCRP: highly sensitive C-reactive protein; IL-6: interleukin 6; HbA1c: glycated haemoglobin; HOMA-IR: homeostatic model assessment for measuring insulin resistance.
Baseline compared to 6 months: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$
Baseline compared to 12 months: $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$
6 months compared to 12 months: $^{\S}P<0.05$; $^{\S\S}P<0.01$; $^{\S\S\S}P<0.001$

Chapter 5: Enhancements in High-Density Lipoprotein Functionality after Bariatric Surgery are related to reductions in adipose tissue and systemic inflammatory cytokines

To be submitted for publication

Author's Contribution: Safwaan Adam researched data (patient recruitment, clinical assessments, venous blood sample collection, assisted with adipose tissue collection and serum, plasma and adipose tissue separation for storage), analysed and interpreted data. In addition, Safwaan Adam researched the available literature and wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript. The manuscript formed the basis of this chapter.

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Enhancements in High-Density Lipoprotein Functionality after Bariatric Surgery are related to reductions in adipose tissue and systemic inflammatory cytokines

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Abstract

Background:

Emerging evidence suggests an association between impaired high-density lipoprotein (HDL) functionality and cardiovascular disease (CVD). HDL is essential for reverse cholesterol transport (RCT). Additionally, HDL reduces inflammation and oxidative stress, principally mediated by paraoxonase-1 (PON1). RCT depends on HDL's capacity to accept cholesterol (cholesterol efflux capacity [CEC]) and active transport through the channels ATP-binding-cassette (ABC-) A1, G1 and scavenger receptor-B1 (SR-B1). We studied the impact of bariatric surgery (BS) on RCT and other functions of HDL.

Methods:

Biomarkers associated with increased CVD risk including tumour necrosis factor-α (TNF-α), high-sensitivity C-reactive protein (hsCRP), myeloperoxidase mass (MPO), PON1 activity and CEC *in vitro* were measured in 50 patients before, 6 and 12 months post-operatively. Measured biomarkers were compared to an overweight but otherwise healthy (mean body mass index [BMI] 28 kg/m^2) control cohort. Twelve participants had gluteal subcutaneous adipose tissue biopsies (pre- and 6 months post-surgically) in which targeted gene expression (ABCA1, ABCG1, SR-B1, TNF-α) and histological analysis (adipocyte size, macrophage density, TNF-α immunostaining) were undertaken.

Results:

There were significant ($p<0.05$) improvements in BMI, HDL-C, hsCRP, TNFα, MPO mass, PON1 activity and CEC *in vitro*. The biomarkers, although significantly impaired compared to controls pre-operatively, were similar 12-months post-surgery. ABCG1 (*fold-change*, 2.24;p=0.005) gene expression was augmented significantly whereas there was a trend for a favourable non-significant change in TNF-α (*fold-change*, 0.44; p=0.57), SR-B1 (*fold-change*, 1.12; p=0.6) and ABCA1 (*fold-change* 1.34; p=0.05). Adipocyte size ($p<0.0001$), macrophage density (p=0.0067) and TNF-α immunostaining (p=0.0425) were reduced post-operatively. ABCG1 expression was inversely correlated with TNF-α immunostaining (r= -0.71; p=0.03) and linear regression analysis showed that changes in TNF-α significantly affected changes in serum CEC *in vitro* (β= -0.50; p=0.015).

Conclusion:

Bariatric surgery enhances HDL functionality, and this is related to reductions in adipose tissue and systemic inflammation.

Introduction

Obesity is an established independent risk factor for the development of cardiovascular disease (CVD) (1). In addition to the risk conferred by the presence of obesity alone, the excess weight also predisposes an individual to a milieu of other traditional CVD risk factors including hypertension, hyperglycaemia and dyslipidaemia (2). Simultaneously, in obesity, the atherogenic environment is reinforced by increases in inflammation (3) and oxidative stress (4). Reciprocally weight loss (especially after bariatric surgery) has been shown to induce a less atherogenic lipid profile as well as reduce inflammation (3, 5-9). Accordingly, the Swedish Obese Subjects (SOS) study revealed a significant reduction in the incidence of and mortality from CVD over long-term follow-up in individuals who underwent bariatric surgery (10). The authors observed that the reduction in CVD was weight-loss independent (10) thus implying a contribution from other factors, some of which have not yet been fully elucidated.

The dyslipidaemia of obesity has typically been described as causing low high-density lipoprotein cholesterol (HDL-C) levels and hypertriglyceridaemia but additionally having undesirable effects on total cholesterol (TC), apolipoprotein A1 (apoA1), and cholesteryl ester transfer protein (CETP) activity (11). Although HDL-C is inversely related to cardiovascular disease (CVD) risk (12), pharmacologically increasing the levels of HDL-C in three randomised trials did not show CVD benefit (13-15). In a more recent trial using anacetrapib, reductions in CVD were felt to be more likely related to LDL-C lowering as opposed to the increases in HDL-C (16, 17). Additionally, genetic conditions including deficiencies in apoA1, ATP binding cassette (ABC-) A1 and LCAT do not consistently augment CVD risk as might have been expected from the resultant very low levels of HDL-C (18). Similarly, in a previous Mendelian randomisation study genetic predisposition to high HDL-C levels did not offer reciprocal CVD protection (19) whilst mutations in the SCARB1 gene (causing higher HDL-C) led to greater incidence of CVD (20). As a result, the focus is shifting from solely focusing on the HDL cholesterol cargo to its functionality.

HDL particles have important functions including anti-oxidative, anti-inflammatory, anti-glycation and anti-thrombotic activity (21-25). More importantly, however, for CVD protection, HDL is the principal facilitator of the reverse cholesterol transport (RCT) pathway: the process by which excess cholesterol is removed from the body (25, 26). Cholesterol efflux capacity (CEC) of HDL *in vitro* represents the capacity of HDL to accept cholesterol from peripheral cells and this test is validated as an *in vitro* assessment of HDL's function and a predictor of cardiovascular risk in large clinical trials (27). However, RCT is a complex process comprised of passive aqueous diffusion and active transport and exchange facilitated principally by ABCA1, ABCG1, scavenger receptor B1 (SR-B1) and CETP activity (28).

Some studies have supported the hypothesis that impairment in HDL function (rather than HDL-C cargo alone *per se*) confers tendency to atherosclerosis. Khera *et al.* showed that reduction in CEC was associated with both carotid artery and coronary artery atherosclerosis in a study involving 996 patients (27). The large Dallas Heart and EPIC-Norfolk population-based studies also both demonstrated an independent association between CEC and incident CVD events (29, 30). Though HDL is an important component of this system, there are many other aspects of this process (in particular at the cellular level) that requires further investigation.

Despite the mechanism of the RCT (including key transporters involved) having been well described in the literature, a limitation to date is that the major studies using it as a surrogate for HDL function have focused mainly on measurement of CEC *in vitro* (27, 29, 30). The most established method of measuring CEC *in vitro* (using radiolabelled cholesterol to measure cholesterol efflux from J774 macrophages) involves a combination of the known passages (ABCA1, ABCG1, SRB1 and passive diffusion) (27). Other studies have conducted functional assessments of individual pathways (31) using different cells lines (which express differing amounts of the transporter proteins) however these may not have fully reflected efflux through one transporter alone as passive diffusion and other unknown channels may not have been fully accounted for. There is a paucity of data quantifying the effects of an intervention on the transporters involved in RCT.

We sought to establish the effect of bariatric surgery on HDL functionality, especially RCT, by measuring CEC and quantifying the key transporters involved in gluteal subcutaneous tissue and investigated factors that may explain improvements in CEC after bariatric surgery. We also assessed the changes in inflammatory and oxidative stress biomarkers following bariatric surgery.

Methods

Study Design, participant enrolment and assessments

This was an observational cohort study in persons who underwent bariatric surgery at Salford Royal NHS Foundation Trust, a Tier 4 specialist weight management centre in North West England. Ethical approval for the study was granted prior to recruitment by the North West – Greater Manchester Central Research Ethics Committee (reference 11/NW/0731). Healthy controls were volunteers who had no existing medical problems nor were they on any medication for any underlying acute or chronic illness. Informed consent was sought prior to any research activities and patients were seen before, 6 and then 12 months after bariatric surgery. Control patients only attended for one visit. At each visit, in addition to a detailed medical history, anthropometric measurements (including height, weight, waist circumference) and body mass index (BMI) calculations were performed. Fasting venous blood samples were taken for biochemistry measurements. Patients with a history of underlying active inflammatory disease, genetic conditions known to affect lipid profile and active or previous malignancy were excluded from the study. Patients who were using fibrates, highly purified omega-3 polyunsaturated fatty acids, nicotinic acid and proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies therapy were also excluded from the study.

Subcutaneous adipose tissue biopsies and adipose tissue analysis

Twelve participants (within the cohort) also consented to undergoing gluteal subcutaneous adipose tissue biopsies (SAT) biopsies before and after surgery. Histological analysis of the adipose tissue was as previously described (32, 33). RNA was extracted from adipose tissue using the RNeasy lipid tissue mini kit

(Qiagen) and reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). The cDNA concentration and purity was determined using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific). Relative gene expression was determined using a LightCycler 480 machine (Roche) running LightCycler 480 SW 1.5.0 SP3 software. The assays used in this study were Roche RealTime ready single assays (ABCA1, ABCG1, SCARB1 and TNF-α) relative to two control genes (ACTB and RN18S1). All genes were assayed in triplicate with reaction efficiencies of 100% and 50ng of total cDNA was used per reaction for all genes except TNF-α which required 100ng of total cDNA per reaction.

Biochemistry Measurements

Laboratory measurements were done in the Lipid Research Group Laboratory at the University of Manchester. Total cholesterol was measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) method whilst triglyceride measurements were done using the glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase (GPO-PAP) method using reagents from ABX Horiba-UK. HDL-C determination was by using a direct second-generation homogeneous method (Roche Diagnostics). Apolipoprotein A1 (apoA1) was determined using an immunoturbidimetric assay with a Cobas Mira analyser (Horiba ABX Diagnostics, UK). The calibration was traceable to the International Federation of Clinical Chemistry primary standards thereby ensuring compatibility with other published work (34). High-sensitivity C-reactive protein (hsCRP) measurements were done in serum using an in-house, antibody sandwich ELISA technique using anti-human CRP antibodies, calibrators and controls from Abcam (Cambridge, UK). Serum tumour necrosis factor-alpha TNF-α was measured using the DuoSet ELISA development kits from R&D systems. PON1 activity was measured as previously described (35). In summary, serum PON1 was assessed using paraoxon (O, O-Diethyl O-(4-nitrophenyl) phosphate; Sigma-Aldrich Corp. St. Louis, MO, USA) as the substrate and the rate of generation of p-nitrophenol was determined at room temperature over 3 minutes using a continuously reading spectrophotometer (Labsystems multiskan multisoft plate reader from Labsystems, Hampshire, UK) at 405 nm. Plasma myeloperoxidase mass (MPO) was determined using an ELISA kit

from R&D systems. Cholesterol efflux capacity of HDL (CEC) was measured using a previously validated method (36). Briefly, J774A.1 cells were incubated with radiolabelled cholesterol and then incubated with apolipoprotein B-depleted serum for four hours. Following incubation, the cell media were collected, and the cells were washed with phosphate buffered saline (PBS) and then dissolved in 0.5 ml 0.2N NaOH to determine radioactivity. Cellular cholesterol efflux was expressed as the percentage of radioactivity in the medium from the radioactivity in the cells and medium collectively. Cholesterol efflux was linear over 4 hours and with concentration. CEC was then calculated using the following formula:

$$\text{CEC (\%)} = \frac{\text{radioactivity in medium}}{\text{Radioactivity in cell + radioactivity in medium}} \times 100$$

To calculate CEC pre- and post-operatively, efflux was subtracted to serum-free serum media (as control). CETP activity was measured using a modification to the Stokke Norum method using the participant's own lipoprotein as substrates (37). All tests were done in duplicate to ensure accuracy.

Glycated haemoglobin (HbA1c) measurements were done according to routine laboratory procedures in the Department of Clinical Biochemistry at Manchester University NHS Foundation Trust.

Statistical Analysis

SPSS for Windows (Version 23.0, IBM, SPSS Statistics, Armonk, NY: IBM Corp.) and GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California, USA) were used for all statistical analyses and figure preparation. Tests for normality were done using the Shapiro-Wilk test, visualisation of histograms and Q-Q plots. To compare differences for the variables measured in adipose tissue (two time points), paired t-tests were done for parametric data whilst the Wilcoxon matched-pairs signed rank test was used for non-parametric data. When more than two time points were being compared (biochemistry parameters), one-way ANOVA (paired and unpaired test as appropriate) was used for parametric data. The Kruskal-Wallis test was used for unpaired non-parametric data whilst Friedman's Two-Way Analysis of Variance by Ranks was used for paired non-parametric data. Specific post-hoc pairwise comparisons were done using the Bonferroni correction and Dunn's Test via an automated adjustment method built into GraphPad Prism and SPSS. The adjusted McNemar's test was used to compare paired categorical

variables. Correlation analysis was done using Pearson's test for parametric and Spearman's test for non-parametric data. To obtain values for correlation analysis, individual participant's gene expression fold change was calculated using the the $2^{-\Delta CT}$ at each time point for each individual patient (38, 39). When assessing for changes and statistical significance of gene expression changes between the pre-operative and post-operative states, we used the ratio of pre- and post-surgery $2^{-\Delta CT}$ calculations as suggested in *example 5* of a previous publication by Schmittgen and Livak (39). Stepwise regression analysis was carried out to understand predictive variables in a model predicting influencing factors of percentage change in CEC. Variables were chosen based on pre-identified possible factors in a prior univariate analysis assessing changes in CEC. Statistical significance was considered to be present when either the p-value or SPSS/GraphPad Prism adjusted p-value was <0.05.

Results

Changes in parameters following bariatric surgery

50 participants (12 men) were enrolled into the study; 39 patients underwent Roux-en-Y Gastric Bypass (RYGB) whilst 11 patients had laparoscopic sleeve gastrectomy (LSG). There were significant reductions in BMI, waist circumference, glycated haemoglobin, triglycerides, hsCRP, TNF-α, MPO mass and CETP activity (Figure 1; Table 1). Additionally, there were significant increases in HDL-C, PON1 activity and CEC (Figure 1).

Comparisons between patients with and without T2DM and statin use respectively of HDL-C, ApoA1, hsCRP, TNF-α, PON1 activity, MPO mass, CEC and CETP did not reveal any significant differences in these variables. Post-operatively, there were no differences (both 12-month post-operative value and percentage change) in either CEC, PON1 activity, hsCRP, TNF-α or MPO mass between those who had RYGB and LSG.

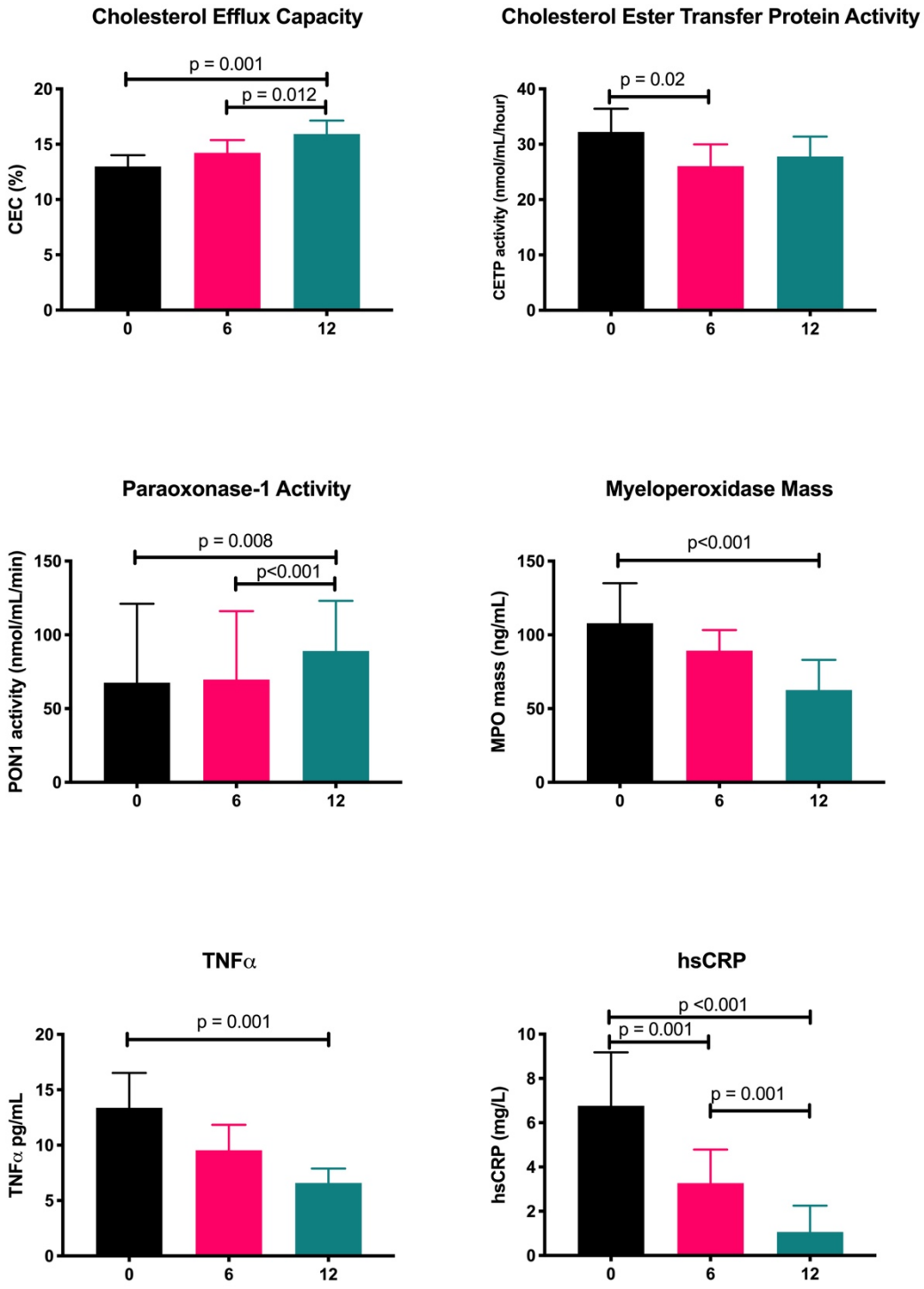


Figure 1. Changes in variables following bariatric surgery.

Bars represent median value and error bars correspond to 95% C.I. of the median. Horizontal axis represents time since bariatric surgery. CEC: cholesterol efflux capacity; CETP: cholesterol ester transfer protein; PON1: paraoxonase-1; MPO: myeloperoxidase; TNF-α: tumour necrosis factor-α; hsCRP: high-sensitivity C-Reactive protein.

Table 1: Changes in parameters before and after bariatric surgery

N=50	*Baseline*	*6 Months*	*12 Months*	*P*
Age	49.9 (9.3)			
BMI (kg/m^2)	49.3 (45.8-57.4)	37.4‡ (33.5-44.1)	35.0*§ (30-39.5)	**<0.0001**
WC (cm)	137.4 (130-150)	113.0‡ (106-130)	104.5*§ (97-120)	**<0.0001**
Type 2 Diabetes (%)	24/50 (48)		5/50 (10)	**0.001**
Statin Use (%) †	33/50 (66)		23/50 (36)	**0.004**
HbA1c (mmol/mol)	45.4 (41.3-56.3)	38.9‡ (33.9-42.3)	36.0*§ (33-38.8)	**<0.0001**
Total Cholesterol (mmol/L)	4.54 (1.31)	4.62 (1.30)	4.44 (0.85)	0.349
LDL-C (mmol/L)	2.37 (1.95-3.14)	2.63 (1.82-3.62)	2.42 (1.99-3.15)	0.089
Triglycerides (mmol/L)	1.51 (1.16-2.07)	1.41 (1.01-1.67)	1.07*§ (0.9-1.40)	**<0.0001**
HDL-C (mmol/L)	1.11 (0.91-1.32)	1.16 (0.93-1.44)	1.29*§ (1.05-1.56)	**<0.0001**
ApoA1 (g/L)	1.26 (1.16-1.38)	1.20 (1.07-1.42)	1.25 (1.11-1.41)	0.267
hsCRP (mg/L)	6.52 (3.99-11.6)	3.27‡ (1.23-7.71)	1.07*§ (0.4-3.2)	**<0.0001**
TNF-α (pg/ml)	8.4 (5.2-26.3)	6.6 (4.8-10.1)	5.7* (4-7.8)	**0.001**
PON1 activity (nmol/ml/min)	67.50 (41.3-171)	69.70 (44.4-163)	89.05*§ (50-157)	**<0.0001**
Myeloperoxidase Mass (ng/ml)	107.9 (83.4-147)	87.3 (66.4-117)	62.6* (47.7-97.1)	**<0.0001**
CEC (%)	13.0 (3.59)	14.2 (3.89)	15.9*§ (4.3)	**<0.0001**
CETP Activity (nmol/ml/hr)	32.2 (11.1)	26.1‡ (11.6)	27.8 (10.8)	**0.02**
HOMA-IR	5.66 (4.05-10.15)	2.55‡ (1.62-4.09)	1.78* (1.17-2.78)	**<0.0001**

* SPSS Bonferroni Adjusted p<0.05: twelve-month value vs. baseline value. § SPSS Bonferroni Adjusted p<0.05: twelve-month value vs. six-month value. ‡ SPSS Bonferroni Adjusted p<0.05: Six-month value vs. baseline value. Data represented as mean (±standard deviation) for parametric data and median (interquartile range) for non-parametric data. BMI: body mass index; WC: waist circumference; HbA1c: glycated haemoglobin; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; ApoA1: apolipoprotein A1; hsCRP: high-sensitivity C-reactive protein; TNF-α: tumour necrosis factor α; PON1: paraoxonase-1; CEC: cholesterol efflux capacity; CETP: cholesterol ester transfer protein; HOMA-IR: Homeostatic Model of Assessment for Insulin Resistance. †No alternate lipid-modifying treatment was used (including over the counter)

Comparison between bariatric surgical patients and controls

Twenty volunteers were recruited to act as controls. There were significant differences between the control cohort and the obese (surgical cohort) patients' baseline measures in BMI, waist circumference, HbA1c, triglycerides, PON1 activity, hsCRP, MPO and CEC (Supplementary Table 1; Figure 2). In each of those variables the control cohort had a healthier profile. Six-months post-operatively, significant differences between controls and surgical patients were seen in BMI, waist circumference, apoA1 levels, PON1 activity and MPO mass (Supplementary Table 1; Figure 2); the controls continued to have a more favourable profile of those variables. At twelve months post-operatively, the only significant difference between the surgical cohort and control cohort was in apoA1 levels (lower in post-operative patients compared to controls).

Supplementary Table 1: Comparison between surgical cohort and control cohort

	Bariatric Surgery Cohort (n=50)			Controls (n=20)
	Baseline	***6 Months***	***12 Months***	***Controls***
Age	49.9 (9.3)			**55.3 (16.2)**
BMI (kg/m^2)	49.3 (45.8-57.4)	37.4 (33.5-44.1)	35.0 (30.1-39.5)	**28.2*§ (26.0-32.5)**
WC (cm)	137.4 (130-150)	113.0 (106-130)	104.5 (96.5-120)	**100.0*§ (92.0-110.0)**
HbA1c (mmol/mol)	45.4 (41.3-56.3)	38.9 (33.9-42.3)	36.0 (33-38.8)	**42.0* (33.0-43.0)**
Total Cholesterol (mmol/L)	4.54 (1.31)	4.62 (1.30)	4.44 (0.85)	**4.94 (1.24)**
Triglycerides (mmol/L)	1.51 (1.16-2.07)	1.41 (1.01-1.67)	1.07 (0.92-1.40)	**1.05* (0.67-1.67)**
LDL-C (mmol/L)	2.37 (1.95-3.14)	2.63 (1.82-3.62)	2.42 (1.99-3.15)	**2.85 (2.43-3.89)**
HDL-C (mmol/L)	1.11 (0.91-1.32)	1.16 (0.93-1.44)	1.29 (1.05-1.56)	**1.23 (1.06-1.48)**
ApoA1 (g/L)	1.26 (1.16-1.38)	1.20 (1.07-1.42)	1.25 (1.11-1.41)	**1.37§‡ (1.22-1.59)**
hsCRP (mg/L)	6.52 (3.99-11.6)	3.27 (1.23-7.71)	1.065 (0.44-3.23)	**1.85* (1.07-2.73)**
TNF-α (pg/ml)	8.4 (5.2-26.3)	6.6 (4.8-10.1)	5.7 (4-7.8)	**6.6 (5.4-7.9)**
PON1 activity (nmol/ml/min)	67.50 (41.3-171)	69.70 (44.4-163)	89.05 (49.9-157)	**116.8*§ (73.4-287.6)**
Myeloperoxidase Mass (ng/ml)	107.9 (83.4-147)	87.3 (66.4-117)	62.6 (47.7-97.1)	**55.4*§ (42.4-67.4)**
CEC (%)	13.0 (3.59)	14.2 (3.89)	15.9 (4.3)	**15.8* (2.8)**
HOMA-IR	5.66 (4.05-10.15)	2.55 (1.62-4.09)	1.78 (1.17-2.78)	**2.50* (1.50-4.87)**

*p<0.05 controls vs baseline. §p<0.05 controls vs 6 months. ‡p<0.05 controls vs 12 months.

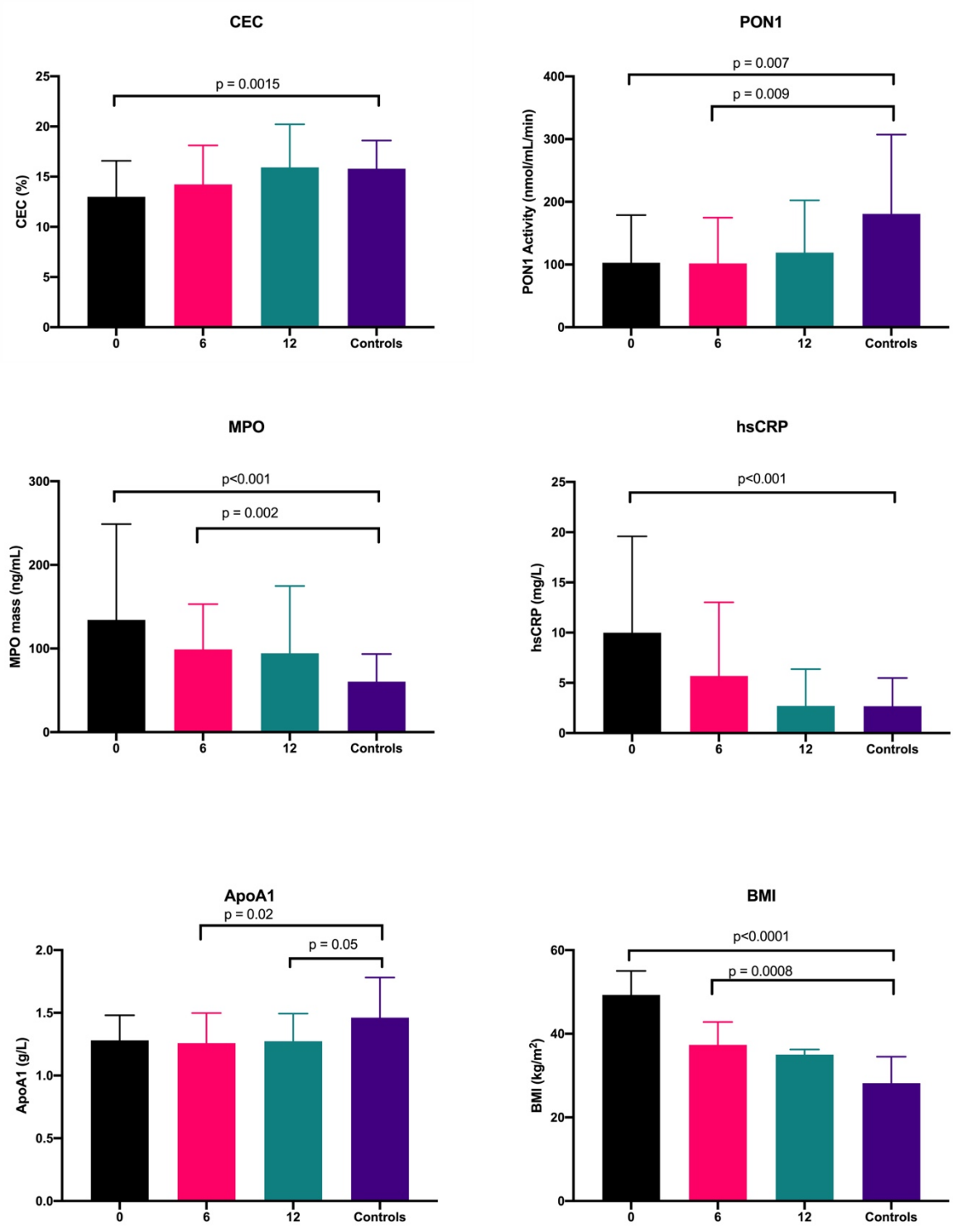


Figure 2. Comparison of key variables between controls and surgical cohort

Specific comparisons of variables between control patients and obese patients at baseline and six and twelve months post-operatively. Bars represent median value and error bars correspond to 95% C.I. of the median. Horizontal axis represents time since bariatric surgery. CEC: cholesterol efflux capacity; CETP: cholesterol ester transfer protein; PON1: paraoxonase-1; MPO: myeloperoxidase; TNF: tumour necrosis factor; hsCRP: high-sensitivity C-Reactive protein.

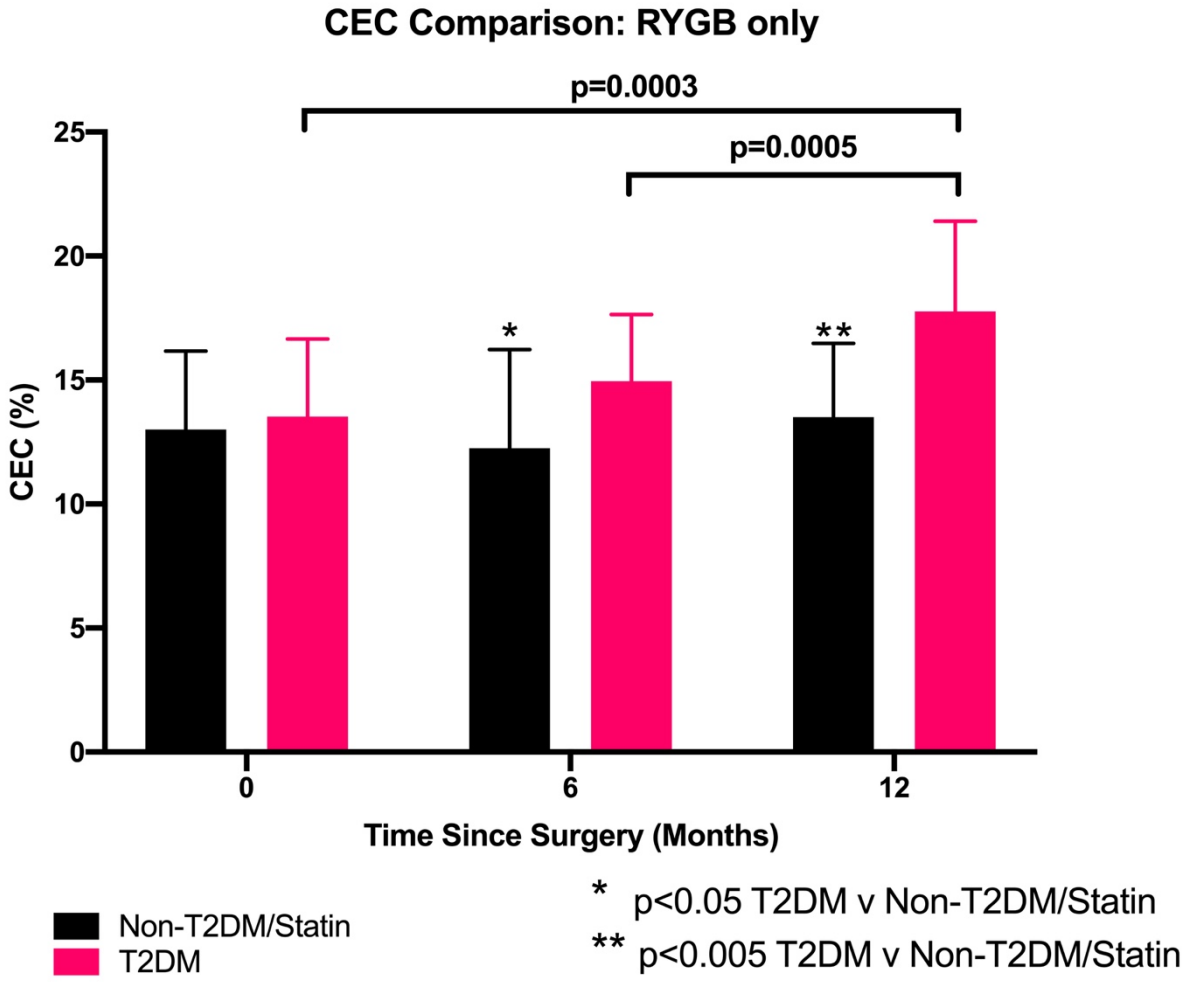
Does Roux-en-Y Gastric Bypass improve HDL functionality?

To assess for the effect of RYGB on CEC, the 39 patients who underwent the procedure were analysed separately. There was a significant improvement in both CEC ($p<0.001$) and PON1 activity ($p=0.003$). CEC was significantly enhanced ($p<0.001$) between baseline and 12-months postoperatively (mean 13.1% [3.7] to 16.1% [4.0]). There was also a significant improvement ($p<0.001$) in CEC between 6 and 12 months post-RYGB (mean 13.8% [3.6] to 16.1% [4.0]). PON1 activity also changed significantly ($p=0.03$) from a baseline mean of 104 (79.2) to 123 (86.4) nmol/ml/min twelve months following RYGB. Similarly, mean PON1 activity was augmented between six (104 [77.0]) and twelve months post-RYGB ($p=0.002$).

A further sub-analysis of patients who underwent RYGB comprised of separating those with T2DM and comparing them to patients without a history of T2DM or statin use. Fifteen patients neither used statin treatment nor were known to have T2DM, whilst 19 patients were known to have T2DM (all statin treated and unchanged following surgery). The principal focus was to assess for pre- and post-operative differences within and between the two subgroups. We found that the group who were neither treated with statins nor had T2DM, although did not have significantly different baseline CEC measures, had significantly lower ($p<0.05$) CEC than the group with T2DM (Supplementary Figure 1). Furthermore, CEC did not change significantly in the non-T2DM/non-statin group between baseline and post-operative measures.

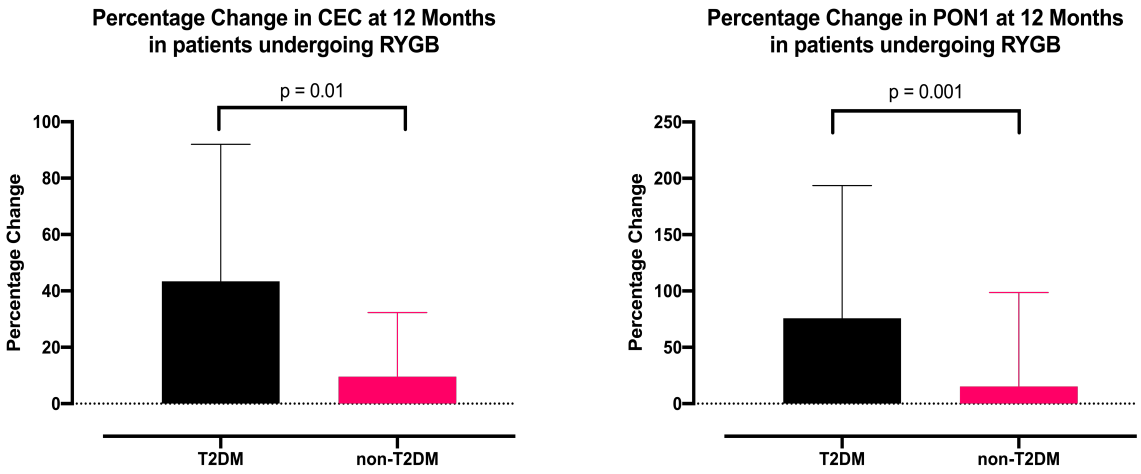
In the group comprising patients with T2DM only, there were significant improvements in CEC between baseline and 12-month post-operative measures and between the 6 month and 12-month post-operative measures (Supplementary Figure 1).

Moreover, there were significant differences ($p=0.01$) between the percentage change in CEC and PON1 activity 12 months post-bariatric surgery between groups (Supplementary Figure 2). There were no other significant differences between the non-T2DM and T2DM groups (who underwent RYGB) in changes in other variables at either six or twelve-months post-RYGB. Furthermore, there was no correlation between changes in CEC, PON1 activity and other variables in either group respectively.



Supplementary Figure 1. Comparison of CEC in groups (T2DM v non-T2DM) following RYGB

CEC at baseline, 6 and 12 months post-RYGB in respective sub-groups (T2DM v non-T2DM/statin). All patients with T2DM were statin treated. Bars represent mean and error bars represent standard deviation. There were significant differences in six- and twelve-month postoperative values between the T2DM and non-T2DM/statin group. Within the T2DM group, there was a significant improvement in CEC from baseline to twelve-months post-RYGB as well as between six- and twelve-months post-surgery. In the non-T2DM group there were no significant increases in CEC. CEC: cholesterol efflux capacity; T2DM: Type 2 Diabetes; RYGB: Roux-en-Y Gastric Bypass.



Supplementary Figure 2. Comparison of percentage change between groups following RYGB

There were significant differences between the percentage change (from baseline to twelve-months post-RYGB) in the T2DM group and the non-T2DM group with greater percentage changes in the T2DM group.

Changes in adipose tissue gene expression and histology before and after bariatric surgery

In the twelve participants who underwent gluteal SAT biopsies, there was a significant increase in ABCG1 expression post-operatively (Table 3). Changes in the gene expression of ABCA1, SCARB1 and TNF-α were not statistically significant (Table 2). In addition, six months after bariatric surgery, on histological evaluation, there were significant reductions in adipocyte area, the percentage area of TNF-α staining and the number of macrophages within the adipose tissue samples (Table 3). The biochemical parameters for this subset of 12 patients only showed significant reductions ($p<0.001$) in hsCRP and CETP activity six months post-operatively (when biopsy was done). There were no relationships between biochemical parameters and either histological findings or gene expression measures.

Table 2.Changes in gene expression post-operatively

Gene (n =12)	**Fold Change (before and 6 months after surgery)**	***p***
ABCA1	1.34	0.070
ABCG1	2.24	**0.005**
SCARB1	1.12	0.630
TNF-α	0.44	0.270

The changes in gene expression between the pre- and 6-months post-operative states are shown in Table 2. ABCG1 was the only gene that changed significantly with non-significant favourable changes seen in ABCA1, SCARB1 and TNF-α gene expression. ABCA1: ATP-binding cassette A1; ABCG1: ATP-binding cassette G1; SCARB1: scavenger receptor B1; TNF-α: tumour necrosis factor alpha.

Table 3. Changes in histological parameters of gluteal adipose tissue before and after surgery.

Parameter (n = 12)	**Baseline**	**6 months post-surgery**	***p***
Adipocyte Area (μm^2)	7721 (1471)	3895 (814.8)	**<0.0001**
Macrophage density (per mm^2)	6.93 (4.6-10.9)	3.61 (1.81-4.89)	**0.005**
*TNF-α immunostaining**	1.13 (0.58-1.87)	0.59 (0.44-0.86)	**0.043**

**Depicted as percentage of the area analysed when measured at the same grey pixel range. TNF-α: tumour necrosis factor-α. Following bariatric surgery, there were significant reductions in the size of adipocytes, the macrophage density measured, and the amount of tissue stained by TNFα.*

Pre- and Post-Operative Relationships Between Variables

Cholesterol Efflux Capacity

At baseline there were no significant relationships seen between CEC and other variables. Six months post-operatively, there was a significant inverse correlation between serum TNF-α concentration and CEC (r -0.42; p=0.004) but not with other variables. At twelve months post-bariatric surgery, the inverse relationship between TNF-α and CEC remained (r=-0.32; p=0.027), albeit weaker (compared to the 6-month relationship). There was also a moderate inverse correlation between percentage changes (from baseline to 12-months post-operative) in CEC and HbA1c (r=-0.33; p=0.02) (Figure 3A).

PON1 activity

At baseline, there was a significant correlation between PON1 activity and HDL-C (r=0.40; p=0.004) however these variables were not related post-operatively. PON1 activity was not associated with other factors post-operatively. Post-operative percentage changes in HDL-C and PON1 activity (r=0.35; p=0.014) were significantly correlated (Figure 3A).

Predictors of change in CEC

There was a significant inverse correlation between percentage changes in HbA1c and CEC (percentage change between baseline and 12 months post-operative). To further examine any potential relationships, stepwise multiple regression analysis was done to assess predictive factors for percentage changes in CEC. There were significant inverse associations with percentage changes in HbA1c and circulating TNF-α concentration (Table 4).

Table 4. Factors predicting post-operative percentage change in cholesterol efflux capacity

Predictive Factor	Unstandardised B	Beta	p
% change in HbA1c	-0.65	**-0.332**	**0.049**
% change TNF-α	-0.26	**-0.501**	**0.015**
% change Triglycerides	0.07	0.107	0.603
% change PON1 activity	-0.09	-0.296	0.082
% change BMI	0.945	0.239	0.171

R^2 =0.27; p=0.04

Stepwise regression model with variables predicting post-operative percentage change in CEC. HbA1c and TNF-α were the only significant predictors. HbA1c: glycated haemoglobin; TNF-α: tumour necrosis factor-α; PON1: paraoxonase-1; BMI: body mass index.

Relationships between post-operative percentage change between other variables

There were significant relationships between percentage changes in triglycerides and percentage changes in HDL-C (r=-0.42; p=0.002), total cholesterol (r=0.37; p=0.008) and apoA1 (r=-0.35; p=0.013). Percentage changes in HDL-C also significantly correlated with percentage changes in apoA1 (r=0.64; p<0.001) and HbA1c (r=-0.31; p=0.032). There was also a significant relationship between percentages changes in total cholesterol and MPO mass (r=0.30; p=0.045) (Figure 3A).

Adipose Tissue Gene Expression and Histology

Relationships within adipose tissue parameters, gene expression and histology, were also sought at baseline, six months post-operatively and between proportionate changes in variables. These are represented in Figures 3B (pre-operative), 3C (post-operative) and 3D (changes) respectively.

At baseline there were significant associations between ABCA1 and SCARB1 gene expression (r=0.91; p <0.0001) as well as a significant inverse association between SCARB1 gene expression and TNF-α gene expression (r=-0.64; p = 0.03). TNF-α gene expression was also directly related to adipocyte area (r=0.69; p = 0.02) and showed an inverse relationship with ABCA1 gene expression (-0.58; p=0.049). TNF-α immunostaining was also found to have a direct relationship with adipocyte area (r=0.66; p=0.03). At six months post-operatively, the only significant relationship was between ABCA1 and ABCG1 gene expression (r=0.87; p=0.0005).

When assessing relationships between changes in variables in adipose tissue, the fold change in ABCA1 correlated significantly directly with both fold changes in the SCARB1 gene (r=0.63; p=0.03) and inversely with the TNF-α fold changes gene (r=-0.64; p=0.04). ABCG1 gene expression correlated inversely with TNF-α immunostaining (r=-0.71; p=0.03).

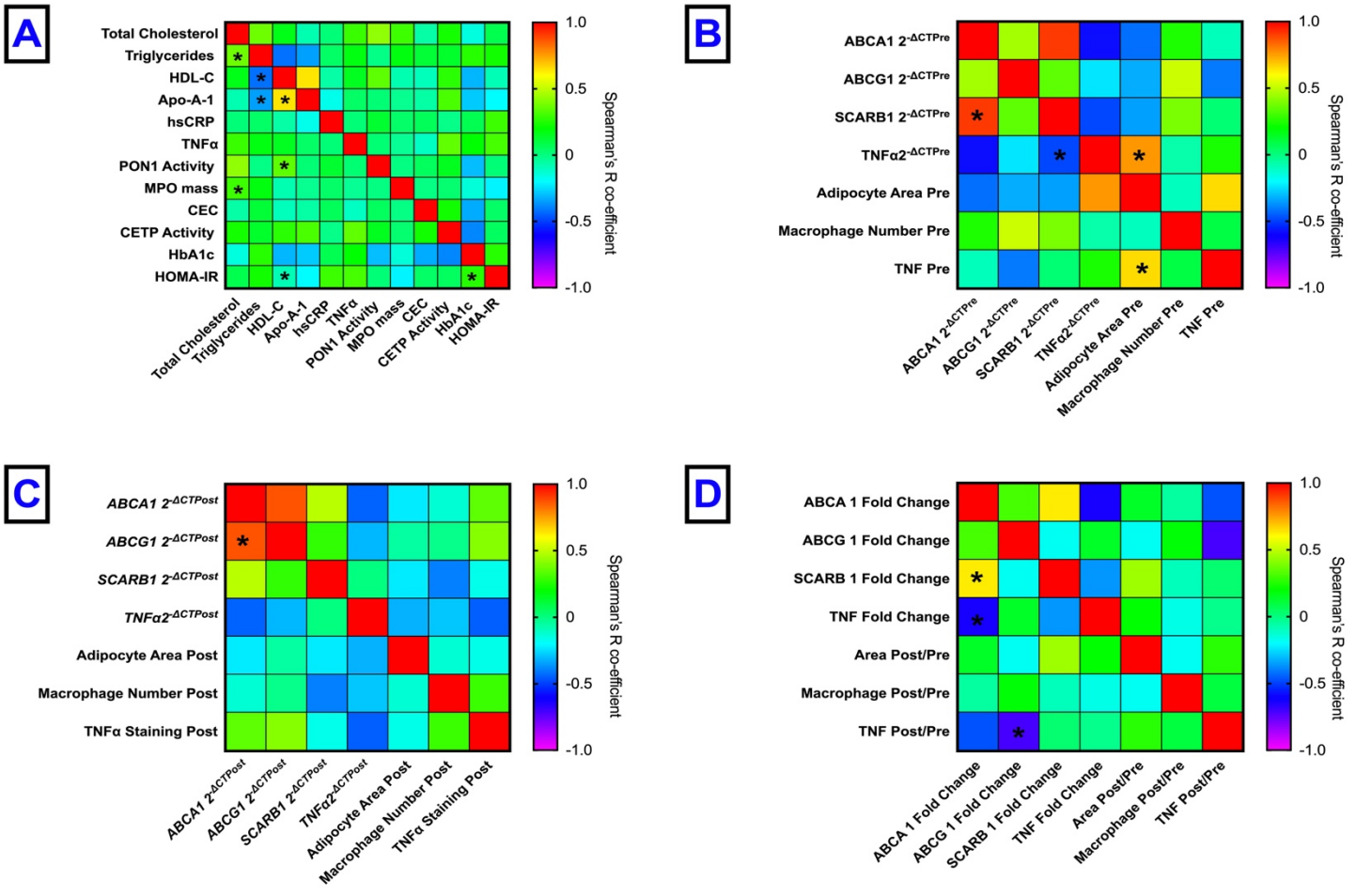


Figure 3. Correlation matrix of A) percentage changes in variables from baseline to 12-months post-surgery B) baseline adipose tissue factors C) six-months post-operative relationships D) markers of changes in adipose tissue.

***p<0.05 denoting a significant relationship**

The matrices demonstrate the strength and significance of relationship between different variables, either as measures of changes in variables (panel A and panel D) or at baseline (panel B) and post-operatively (panel C)

Discussion:

In this study we report a number of novel findings that will help to understand how HDL functionality and inflammation interact and affects cardiometabolic risk profile in a cohort of patients who had undergone bariatric surgery. Firstly, RCT is enhanced as demonstrated by the improvement in CEC *in vitro* and underlined by quantitative increases in ABCG1 gene expression in adipose tissue. Secondly, there is an increase in PON1 activity which is a key mediator of HDL's anti-inflammatory and anti-oxidative stress functions. Additionally, we show that after bariatric surgery, there is a reduction of systemic and local adipose tissue inflammation resulting in a healthier profile in adipose tissue post-operatively. Moreover, we showed that reductions in inflammation in adipose tissue and systemically were associated with increase in ABCG1 and serum CEC respectively following bariatric surgery. Although we did not identify any relationship between CEC and HDL-C, improvements in PON1 activity did show a relationship to post-operative improvements in HDL-C. Our study also found, for the first time, that improvement in glycaemia is an important contributor to enhancement of CEC post-bariatric surgery. Furthermore, following bariatric surgery, severely obese patients whose pre-operative measures were significantly abnormal compared to a control cohort, achieved levels of CEC, PON1 activity, CRP, HOMA-IR and HbA1c that matched the controls' results following bariatric surgery (Figure 2).

Our findings of increases in CEC and ABCG1 substantiate a previous study by Aron-Wisnewksy *et al.* which displayed a functional enhancement of ABCG1 mediated cholesterol efflux 6 months after RYGB (31). The authors suggested that the improvement in ABCG1 mediated efflux *in vitro* (with commercially available macrophages) was a consequence of an increase in HDL2 particles as opposed to an increase in ABCG1 quantities, the latter of which we have now shown to occur at a cellular level in vivo for the first time. We did not however measure HDL particle size and so it is possible that there is more than one mechanism responsible for improvements in ABCG1 related cholesterol efflux.

Aron-Wisnewsky and colleagues also showed that SR-B1 mediated cholesterol efflux was enhanced and ABCA1-mediated efflux *in vitro* was functionally reduced 6 months post-surgery (31). Heffron *et al.* also recently showed a reduction in ABCA1-mediated efflux *in vitro* at 6 months following RYGB (40). In our study, where the

predominant procedure was RYGB, we showed that the principal change in CEC happened between the six and twelve-month timepoint post-operatively which may complement the findings of that study. Furthermore, Heffron and colleagues showed that LSG was superior to RYGB in enhancing cAMP and ABCG1-mediated cholesterol efflux at both six and twelve months post-operatively (40). In contrast, we show marked improvements in CEC in patients who had RYGB. Importantly, we did not find any procedure related differences in the baseline, post-operative or percentage changes in CEC. Notably, in both aforementioned studies, they did not recruit patients with diabetes or statin treatment (31, 40). This is a key difference between our cohort and the other studies and indeed may represent that in patients with T2DM, RYGB achieves improvements in CEC that would not otherwise be seen in patients without diabetes and not on statins. Our sub-analysis shows that the gradient of change of CEC in patients with T2DM was significantly greater than patients without T2DM in patients who underwent RYGB. Indeed, in regression analysis we show changes in HbA1c to be a predictive factor of an improvement in CEC. Additionally, patients who underwent RYGB with a prior history of T2DM also showed a greater percentage improvement in PON1 activity compared to those without T2DM. These findings are important when recognising that in those with T2DM, RYGB may confer additional metabolic benefit and therefore deserves increased consideration.

In our study, although quantitative changes in the gene expression of ABCG1 was statistically significant, changes in the expression of ABCA1 ($p=0.07$) and SCARB1 ($p=0.63$) were not. A different study which assessed differences in ABCA1 and ABCG1 expression in peripheral blood monocytes between people with and without metabolic syndrome found differences between ABCG1 expression but not for ABCA1 (41). In conjunction with our findings, this may suggest that obesity and weight loss have a greater impact on ABCG1 (in the RCT pathway) rather than ABCA1 and this again corroborates with findings from previous studies in patients post-bariatric surgery in which ABCG1 mediated efflux *in vitro* was most enhanced (31, 40). Interestingly, a reduction in ABCG1 has been implicated in atherosclerosis (42) and a propensity towards myocardial infarction in persons with a functional variant (g.-376C>T) of the gene (43).

ABCG1 in adipocytes has previously been thought to exert different actions. It has been implicated in increasing adipocyte size (via lipoprotein lipase driven triglyceride accumulation) and therefore been proposed as a therapeutic target in obesity (44, 45). Contrarily, other studies, like this one, have shown an increase ABCG1 after weight loss (46). This may symbolise, as has previously been speculated, a potentially reversible nature to how ABCG1 functions in an adipocyte (45). Beyond a certain fat content, ABCG1 is thought to promote adipocyte growth whilst alternatively below a different fat threshold it functions optimally in RCT and reduces adipocyte size (as may be the case with weight loss) (45). Additionally, it may represent an alteration of its action due to other factors within the adipocyte including inflammation and indeed we show a strong inverse relationship between ABCG1 gene expression and TNF-α staining in adipose tissue. Notably, a previous study by Umemoto and colleagues showed that by promoting cholesterol efflux in adipose tissue, adipose tissue inflammation was reduced and that ABCG1 was potentially a key mediator in this process (47). Furthermore, a recent epigenome-wide association study analysing the influence of obesity on DNA methylation (n>5000) reported a particular susceptibility of ABCG1 to DNA methylation in relation to BMI (48). This further emphasises the likely fluctuant nature of ABCG1 in states of obesity compared to weight-loss.

PON1, an esterase enzyme which is bound to HDL-C has been shown to be important in preventing oxidation of lipoproteins and therefore atheroprotective (49). In support of this, a previous meta-analysis showed that patients with reduced PON1 activity had a greater incidence of coronary disease (50). However, in a recent parallel population-based study (n=6902) and meta-analysis (pooled n=15064) of previously published prospective studies, Kunutsor *et al.* concluded that when adjusting for HDL-C, altered PON1 levels did not confer additional CVD risk (51).

CETP activity was reduced post-operatively; indeed, previous studies have suggested that a decline in CETP activity leads to an alteration in the HDL particle profile (with an inclination to larger, less atherogenic HDL particles) post-bariatric surgery (31, 52). Although we did not directly measure HDL's anti-inflammatory and anti-oxidative stress functions, another study showed specific weight-independent improvements in these aspects of HDL functionality following bariatric surgery (53).

We also demonstrate reductions in traditional CVD risk factors which include the obesity itself, hypertension, hyperlipidaemia and dysglycaemia, which is in accordance with previous studies and meta-analysis (5, 6, 8, 54, 55).
Overall our study shows a healthier profile to adipose tissue after bariatric surgery with a reduction in both adipocyte size and inflammation. Additionally, we also displayed a favourable change in the nature of adipose tissue after bariatric surgery. There were reductions in both adipocyte size and adipocyte inflammation, with reduction in the number of macrophages and area stained by TNF-α histologically. Our study also shows a marked reduction in systemic inflammation as well as a reduction in oxidative stress biomarkers, especially 12 months after bariatric surgery. A recent randomised trial showed reduction in CVD events when using an anti-inflammatory agent (56) and reductions in inflammation are likely to be a key mechanism by which CVD incidence is decreased after bariatric surgery. Moreover, reductions in adipose tissue inflammation can have extended benefits and we have previously shown a related improvement in perivascular adipose tissue function after bariatric surgery (33).

In the recent large randomised control trials, there was a focus on pharmacologically increasing HDL-C (quantitative increases in the HDL-C cargo) without there being any specific focus on HDL functionality. This might be the reason that results from such interventional trials have been disappointing. It is also important to acknowledge that RCT is a complex system and cannot be assessed with CEC of HDL *in vitro* alone and changes affecting this system at cellular level should also be assessed. We show, in this study, that bariatric surgery increases i) the HDL-C cargo, ii) the total capacity of HDL to accept cholesterol (CEC) *in vitro*, unrelated to changes in HDL-C, iii) an improvement in PON1 activity which is a key component of HDL's antioxidant ability and iv) an enhancement of key cellular transporters that are involved in RCT. Bariatric surgery therefore provides an excellent model in which to study the varied functional nature of HDL. Future therapeutic interventions targeting HDL should take all the above into account. Given the complexity of HDL and the RCT system, it is important to aim for a parallel improvement in these different factors in addition to reductions in inflammation, dysglycaemia and oxidative stress in order to achieve desired outcomes. This multimodal improvement in CVD risk

factors, independent of weight-loss, is likely to be the reason that bariatric surgery has shown such impressive reductions in CVD in longitudinal studies (10, 57).

Strengths of our study include a more comprehensive assessment of HDL functionality and especially the RCT both by measuring CEC and gene expression of key transporters in tandem which allowed us to find a key relationship between reduction in inflammation and HDL functionality. To our knowledge, this is the first time this kind of analysis has been done. We have also been able to demonstrate that RYGB enhances CEC in patients with T2DM and not in those without T2DM; this is a novel finding that will need further validation in a larger cohort. This study also potentially provides further insights into the behaviour of ABCG1 in adipose tissue after surgical weight loss, something that has been scarcely investigated in the past.

Limitations include the relatively small sample size which does require our results to be interpreted with caution. The small number of men participating in our study is small and this limits the generalisability of our findings, although the proportion in this study exceeds other published data (relating to CEC after bariatric surgery) which have exclusively been conducted in women. A key limitation of both ours and others' studies, including large outcome studies, relating to CEC is that we do not use the patient's own macrophages to measure CEC and rather rely on an *in vitro* method using J774 cells. Using patients' own macrophages and HDL would provide a better assessment of the RCT system. To our knowledge a validated method using the patients' own macrophages is not currently available. The mean BMI in our control cohort (who were free of co-morbid disease) was in the overweight category however this measurement is very similar to the current mean BMI of the adult population in the United Kingdom (58).

In summary, this study shows, that there is improvement in HDL function and RCT system following bariatric surgery is represented both by functional measures and quantitative improvements in transporters with reductions in inflammation and hyperglycaemia being key mediators.

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Author Contributions

S.A. conducted a literature search, collected data, analysed and interpreted data and wrote the first draft of the manuscript. J.H. reviewed relevant literature, collected data and worked on the first draft of the manuscript. Y.L, T.S., K.S., S.A., S.S., B.A., A.S. collected data and reviewed the manuscript. R.D., B.A. and A.S collected data, interpreted data and reviewed the manuscript. P.D and H.S. designed the study, interpreted data and reviewed the manuscript.

Declaration of Interests

H.S. has received grants and personal fees from Akcea, grants and personal fees from Amgen, grants from Pfizer, grants and personal fees from MSD, personal fees from Sanofi, personal fees from Synageva, personal fees from Takeda, which are all outside of the submitted work. R.M has received grants and personal fees from Pfizer and Novo Nordisk. None of the other authors have any relevant conflicts of interest to declare.

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Chapter 6: Bariatric Surgery Leads to a Reduction in Antibodies to Apolipoprotein A-1: A Prospective Cohort Study

To be submitted for publication

Author's Contribution: Safwaan Adam researched data (patient recruitment, clinical assessments, venous blood sample collection, and serum and plasma separation for storage), analysed and interpreted data. In addition, Safwaan Adam researched the available literature and wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript. The manuscript formed the basis of this chapter.

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Bariatric Surgery Leads to a Reduction in Antibodies to Apolipoprotein A-1: A Prospective Cohort Study

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Abstract

Background:
Autoantibodies against apolipoprotein A-1 (anti-apoA-1 IgG) have been associated with cardiovascular disease (CVD), poorer CV outcomes and all-cause mortality in different settings, as well as with increased coronary artery calcium score in obese individuals. We studied the impact of bariatric surgery (BS) on the presence of circulating anti-apoA-1 IgG antibodies, and evaluated if anti-apoA-1 IgG changes could be associated with specific biological and clinical characteristics changes, including lipid, insulin resistance, inflammatory profile, and percentage of Excess Body Mass Index Loss (%EBMIL).

Methods:
We assessed 55 patients (40 women) before, 6 and 12 months post-operatively. Baseline and post-operative clinical history and measurements of body mass index (BMI), serum cholesterol, triglycerides, high- and low-density lipoprotein cholesterol (HDL-C and LDL-C), apoA-1, highly sensitive C-reactive protein (hsCRP), fasting glucose (FG), glycated haemoglobin (HbA1c) and HOMA-IR were taken at each point. Human Anti-apoA-1 IgG were measured by ELISA.

Results:
The mean age of participants was 50 years. BS significantly improved BMI, %EBMIL triglycerides, HDL-C, apoA-1, hsCRP, HBA1c, FG and HOMA-IR. Baseline anti-apoA-1 IgG seropositivity was 25% and was associated with lower apoA-1 and higher hsCRP levels. One year after BS, anti-apoA-1 IgG seropositivity decreased to 15 % ($p=0.007$) and median anti-apoA-1 IgG values decreased by 67 % ($p<0.001$). Post-operative anti-apoA-1 IgG levels were significantly associated with a decreased EBMIL at 1-year.

Discussion:
Bariatric surgery results in significant reduction in anti-apoA-1 IgG levels, which may be predictive of impaired weight-loss. The exact mechanisms underpinning these results are elusive and require further study before any clinical recommendation can be made.

Introduction

Obesity is an established independent predictor of cardiovascular disease (CVD) (1) and is associated with increased morbidity and mortality (2). Despite increased recognition of the problem, the prevalence of obesity continues to increase worldwide (3). Obesity is characterised by a state of chronic low-grade inflammation, a key component in the atherosclerotic process and the development of metabolic complications (4, 5). Adipocyte hypertrophy (6) marks the beginning of a cascade of complex interlinked pathophysiological processes involving alterations in adipokine production (7), heightened oxidative stress and lipoprotein modification (8), immune system and inflammatory pathway activation (8), and endothelial dysfunction (9); culminating in atherosclerosis and plaque formation.

Over the past decade, autoantibodies against apolipoprotein A-1 (anti-apoA-1 IgG), the principal protein component of high-density lipoprotein (HDL), have emerged as an independent biomarker for cardiovascular disease and mortality (10-12). Anti-apoA-1 IgG exhibits pro-inflammatory properties through interacting with immune receptors (13) and potentially also exerting a negative effect on HDL function (14). It has been shown to mediate the process of atherothrombosis and contribute to plaque instability (15, 16). It is also associated with elevated levels of oxidised low-density lipoprotein (LDL) which is a key component in all stages of atherosclerosis (17). Anti-apoA-1 IgG was initially identified in patients with autoimmune disease and was linked to atherogenesis and adverse cardiovascular outcomes (18, 19). Subsequent studies added further support to the link between anti-apoA-1 IgG and cardiovascular disease. Anti-apoA-1 IgG was established as an independent predictor of cardiovascular outcome following myocardial infarction (10) and elevated autoantibody titres was found to be prevalent among patients with acute coronary syndrome (20). Within the general population, anti-apoA-1 IgG is independently associated with CVD (12) and is an independent predictor of all-cause mortality (11). In obese patients, anti-apoA-1 IgG has been associated with increased coronary calcium score (21).

Bariatric surgery induces sustained weight loss and long-term reduction in CVD, morbidity and overall mortality (22, 23). Some of the metabolic benefits of surgery,

however, are achieved through weight independent mechanisms (24). Post-surgical reductions in markers of inflammation and oxidative stress, and improvements in HDL structure and function have been shown in some studies (25-27). In this study, we sought to determine the impact of bariatric surgery on anti-apoA-1 IgG levels and positivity and its relationship with cardiovascular risk markers.

Methods

Participants and clinical measures

Fifty-five patients with severe obesity who underwent bariatric surgery at the Salford Royal NHS Foundation Trust tertiary weight management centre (Salford, UK) were recruited into the study. Patients with acute coronary syndrome within 6 months, acute or chronic infections, human immunodeficiency virus, autoimmune diseases, history of malignancy and those receiving steroid therapy or history of immunosuppressive therapy (including chemotherapy and radiotherapy) were excluded. Study visits were undertaken at the National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK) at baseline, 6 months and 12 months after surgery. At each visit, a clinical history, medication review and clinical examination which consisted of measuring anthropometric measures was undertaken, including body mass index (BMI). The percentage excess in BMI loss (%EBMIL) was calculated using the difference in proportionate change in the BMI in excess of a normal BMI of 24.9 kg/m^2.
This study was approved by the Greater Manchester Central Research and Ethics Committee. Written informed consent was obtained from all patients prior to participation in this study and all study assessments were conducted in accordance with the 1964 Helsinki declaration.

Sample collection

Venous blood samples were obtained between 0900 and 1100 following an overnight fasting. Serum and EDTA-plasma were isolated by centrifugation at 4°C within 2 hours of collection and aliquots were stored frozen at -80°C until laboratory analyses performed at the end of study.

Biochemical analyses

Total cholesterol (TC) and triglycerides were measured using the cholesterol oxidase phenol 4-aminoantipyrine peroxidase and glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase methods respectively. High-density lipoprotein cholesterol (HDL-C) was assayed using a second-generation homogenous direct

method (Roche Diagnostics, Burgess Hill, UK). Apolipoprotein A-1 (apoA-1) was measured using immunoturbidimetry assays with a Cobas Mira analyser (Horiba ABX Diagnostics, Nottingham, UK). Glucose was measured using glucose oxidase-phenol and 4-aminophenazone and hexokinase method. All these tests were performed on a Randox daytona+ analyser (Randox Laboratories, Crumlin, UK). The laboratory participated in the RIQAS (Randox International Quality Assessment Scheme; Randox Laboratories, Dublin, Ireland) scheme which is CRC calibrated. Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formula.

An in-house, antibody sandwich ELISA technique using anti-human C-reactive protein (CRP) antibodies, calibrators, and controls (Abcam, Cambridge, UK) was used to measure high-sensitivity CRP (hsCRP).

Glycated haemoglobin (HbA1c) was measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust. Insulin was determined using Mercodia ELISA kits (Diagenics Ltd., Milton Keynes, UK). Insulin resistance was quantified using the homeostatic model assessment (HOMA-IR)(28).

Determination of Anti-apoA-1 IgG levels

Anti-apoA-1 IgG autoantibodies were measured as previously described (10, 11, 16). Briefly, Maxisorb plates (Nunc, Glostrup, Denmark) were coated with purified human-derived delipidated apoA-1 (20μg/ml; 50μl/well) for 1 hour at 37°C. After 3 washes, all wells were blocked for 1 hour with 2% bovine serum albumin (BSA) in a phosphate buffer solution (PBS) at 37°C. Samples were then diluted to 1:50 in PBS/BSA 2% solution and incubated for 60 minutes. Samples at the same dilution were also added to non-coated wells to assess individual non-specific binding. After 6 washes, 50μl of signal antibody (alkaline phosphatase-conjugated anti-human IgG; Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:1000 in PBS/BSA 2% solution was added to each well and incubated for 1 hour at 37°C. After a further 6 washes, 50μl of phosphatase substrate *p*-nitrophenyl phosphate disodium (Sigma-Aldrich, St Louis, MO, USA) dissolved in diethanolamine buffer (pH 9.8) was added. Following

an incubation period of 20 minutes at 37°C, each sample was tested in duplicates and optical density (OD) was determined at 405nm (Molecular Devices™ Versa Max, Sunny Vale, CA, USA). The corresponding non-specific binding was subtracted from mean absorbance for each sample. The specificity of detection of this ELISA for lipid-low and unmodified apoA-1 was confirmed previously by conventional saturation tests, and liquid-chromatography coupled to mass spectrometry (29). Close to the cut-off value (0.6 OD), the inter-assay coefficient of variation was 9% (n=5), and the intra-assay CV of 5% (n=5).

The cut off for anti-apoA-1 IgG positivity was determined as previously described (10, 11, 16). The upper reference range was derived from the 97.5th percentile of reference population for 140 healthy blood donors and this corresponded with an OD cut off of 0.64. An index consisting the ratio between sample OD and the positive control OD expressed as a percentage is further calculated to minimise the impact of inter-assay variation. The index value of 37% corresponded with the 97.5th percentile of the normal distribution. Samples with an absorbance value >0.64 OD and an index value ≥37% were considered as seropositive for elevated anti-apoA-1 IgG levels.

Study Endpoints

The first endpoint consisted in measuring the impact of bariatric surgery on anti-apoA-1 IgG median levels and seropositivity.
The second study endpoint consisted in evaluating if anti-apoA-1 IgG changes could be associated with specific biological and clinical characteristics changes during follow-up.
The third study endpoint consisted in evaluating if baseline and/or post-operative anti-apoA-1 IgG levels were associated with weight-loss or 1-year % EBMIL, a key indicator of bariatric success when constantly over 50% on a long-term follow-up (30).

Statistical analyses

Statistical analyses were performed using SPSS for Mac (Version 23.0, IBM SPSS Statistics, Armonk, NY, USA) and figures were produced using GraphPad Prism for Mac (Version 7.00, GraphPad Software, La Jolla, CA, USA). Normality of data distribution was assessed using Shapiro-Wilk test. Results were presented as mean with standard deviation (SD) and median with interquartile range (IQR) as appropriate. Comparison between baseline and post-surgery was undertaken using the paired t-test for normally distributed variables, Wilcoxon signed-rank test for non-normally distributed variables and McNemar test for categorical variables. For comparison between anti-apoA-1 IgG positive and anti-apoA-1 IgG negative groups, the independent samples t-test and Mann-Whitney U test were performed for normally distributed and non-normally distributed data respectively. We also performed a sub-analysis of patients whose antibody status changed from positive to negative and compared them to those patients whose antibody status remained positive. In this sub-analysis, independent t-test and Mann-Whitney test were used depending on whether the variable was parametric or non-parametric. Binary logistic regression was used to determine if baseline and post-operative anti-apoA-1 IgG levels could be associated with an EBMIL over 50% at 1-year, whilst controlling for key confounders. Correlations between variables were assessed using Spearman's analyses. A p-value of <0.05 was considered to be statistically significant.

Results

Prevalence of Anti-apoA-1 IgG Positivity in severe obesity

Of the 55 patients in the study, at baseline, 14 patients (25%) were positive for autoantibodies against apoA-1 (Table 1 and Figure 1a). The patients who were autoantibody positive were found to have significantly lower apoA-1 levels (p=0.02) as well as higher hsCRP levels (p=0.04) but there were no significant differences in the CVD prevalence, Type 2 diabetes prevalence, statin use, age, other lipid parameters, HbA1c, fasting glucose, HOMA-IR or BMI (Table 2).

The effect of bariatric surgery on Anti-apoA-1 IgG levels and autoantibody positivity status

All 55 patients underwent bariatric surgery and were followed up 12 months post-operatively. Thirty-nine of these patients also returned for a 6 monthly follow-up visit as sixteen patients were unable to attend their 6-month visit. Of these 55 patients, 36 underwent Roux-en-Y gastric bypass surgery (RYGB), 11 patients underwent laparoscopic sleeve gastrectomy (LSG) and 8 patients underwent single anastomosis gastric bypass (SAGB). At baseline, 4 out the 55 patients were known to have CVD; 2 of these had an antibody status of positive and 2 were negative for anti-apoA-1 antibodies (Table 1 and Table 2). Pre-operatively, 29 patients were known to have Type 2 diabetes which remained in only six patients 12 months post-operatively (Table 1). There was a significant reduction in the median BMI of the entire cohort (p<0.001), with a mean %EBMIL of 64% (Table 1).

At baseline, 14 patients (25%) were positive for anti-apoA-1 antibodies and this seropositivity prevalence declined to 8 patients (15%) at 12 months post-bariatric surgery (p=0.007) (Table 1; Figure 1a). There was also a significant reduction in anti-apoA-1 IgG levels in the entire cohort at both 6 and 12 months post-operatively (p<0.001) (Table 1; Figure 1b). Additionally, there were significant changes in HDL-C (p<0.001) and apoA-1 (p=0.043). Triglycerides (p<0.001), hsCRP (p<0.001), HbA1c (p<0.001), fasting glucose (p<0.001) and HOMA-IR (p<0.001) decreased significantly post-operatively both at 6 and 12 months (Table 1). No significant

procedure-related effects nor influence of either type 2 diabetes history or statin use was seen in the post-operative results.

Table 1: Pre- and Post-Operative Comparison of Variables in patients at baseline, 6 months and 12 months after bariatric surgery.

Parameter	Baseline (n=55)	6 Months Post-Surgery (n=39)	12 Months Post-Surgery (n=55)	p-value
Age (years)	50 (9)			
Female Gender (%)	73			
BMI (kg/m^2)	48.0 (44.0-56.0)	37.0††† (33.0-43.0)	33.0***§§ (30.0-38.0)	**<0.001**
%EBMIL			64.4 (20.2)	
History of CVD (%)	7			
Type 2 Diabetes (%)	53		11	**<0.001**
Statin Use	62		41	**<0.001**
Anti-ApoA-1 IgG (AU)	0.70 (0.56-0.84)	0.53††† (0.38-0.72)	0.47*** (0.37-0.61)	**<0.001**
Anti-ApoA-1 Positivity (%)	25	22†	15*	**0.007**
Total Cholesterol (mmol/L)	4.05 (3.54-4.66)	4.07 (3.50-4.70)	4.22 (3.62-4.90)	0.332
Triglycerides (mmol/L)	1.46 (1.11-1.87)	1.24 (0.94-1.61)	1.23*** (0.83-1.49)	**<0.001**
HDL-C (mmol/L)	1.02 (0.85-1.19)	1.15 (0.94-1.33)	1.21***§§ (1.00-1.37)	**<0.001**
ApoA1 (g/L)	1.31 (1.19-1.52)	1.30† (1.14-1.45)	1.30 (1.11-1.50)	**0.043**
LDL-C (mmol/L)	2.35 (1.91-2.84)	2.21 (1.74-2.72)	2.33 (1.95-3.19)	0.622
hsCRP (mg/L)	6.81 (3.99-13.4)	3.14 (1.03-5.51)	1.34 (0.43-3.22)	**<0.001**
HbA1c (mmol/mol)	48 (39-56)	37 (33-41)	35 (32-39)	**<0.001**
Fasting Glucose (mmol/L)	5.70 (4.88-6.91)	4.93†† (4.43-5.93)	4.80*** (4.40-5.53)	**<0.001**
HOMA-IR	5.48 (3.41-8.34)	1.95††† (1.15-3.15)	1.69*** (1.17-2.68)	**<0.001**

*p<0.05; **p<0.01; ***p<0.001 on post-hoc testing between baseline and 12 months post-operatively.
†p<0.05; ††p<0.01; †††p<0.001 on post-hoc testing between baseline and 6 months post-operatively.
§p<0.05; §§p<0.01; §§§p<0.01 on post-hoc testing between 6 and 12 months post-operatively.

Age is representative at the time of surgery; BMI: Body Mass Index; CVD: Cardiovascular Disease; HDL-C: High Density Lipoprotein Cholesterol; ApoA1: Apolipoprotein A1; LDL-C: Low Density Lipoprotein Cholesterol; hsCRP: highly sensitive C-reactive Protein; HbA1c: glycated haemoglobin; HOMA-IR: Homeostatic Model of Assessment for Insulin Resistance.

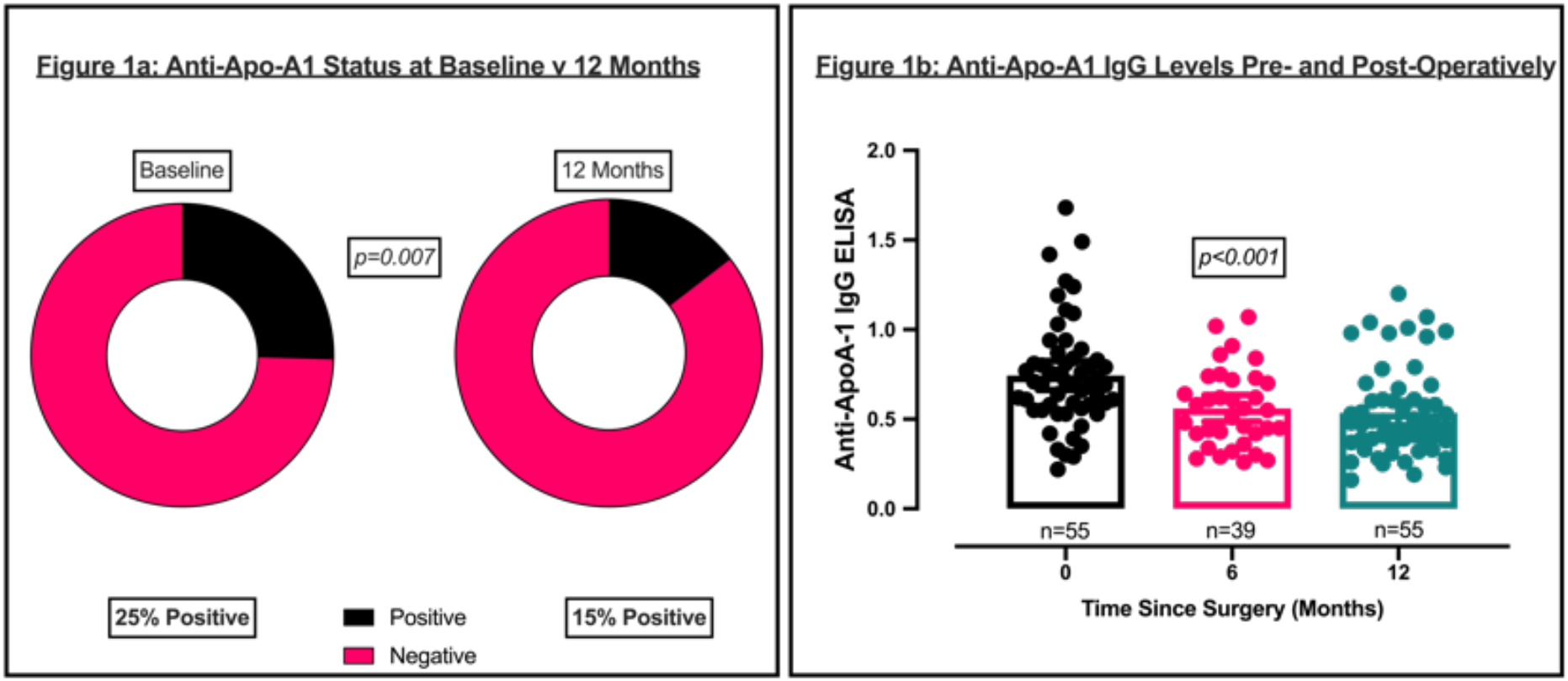

Figure 1. Changes in anti-apoA-1 seropositivity (a) and anti-apoA-1 levels before and after bariatric surgery

Panel (a) shows a significant reduction in anti-apoA-1 seropositivity status 12-months after bariatric surgery. Figure 1(b) shows reductions in antibody titres from baseline to six- and twelve-months following bariatric surgery. Anti-apoA-1: anti-apolipoprotein-A-1

Table 2: Comparison of Variables between patients who tested positive vs negative for Anti-ApoA-1 Antibodies (at baseline)

	Positive (n = 14)	**Negative (n = 41)**	**p**
Female Sex (%)	71	73	1.00
Age (years)	51 (11)	49 (9)	0.679
Diabetes Prevalence (%)	43	56	0.537
CVD Prevalence (%)	14	5	0.266
Anti-ApoA-1 IgG (OD)	1.14 (0.25)	0.61 (0.16)	**<0.001**
Total Cholesterol (mmol/L)	4.17 (3.60-4.66)	4.03 (3.53-4.95)	0.847
Triglycerides (mmol/L)	1.53 (0.77-1.78)	1.45 (1.18-1.94)	0.374
HDL-C (mmol/L)	0.96 (0.84-1.35)	1.03 (0.82-1.19)	0.931
ApoA1 (g/L)	1.27 (1.09-1.41)	1.33 (1.20-1.55)	0.220
LDL-C (mmol/L)	2.44 (1.99-2.94)	2.26 (1.79-2.92)	0.615
hsCRP (mg/L)	10.4 (5.79-17.5)	5.91 (3.43-9.32)	**0.042**
HbA1c (mmol/mol)	47 (42-51)	48 (39-58)	0.862
Fasting Glucose (mmol/L)	5.65 (4.95-6.51)	5.80 (4.82-7.07)	0.517
HOMA-IR (%)	4.30 (3.92-5.74)	5.82 (2.80-9.99)	0.428
BMI (kg/m^2)	47.5 (43.8-56.3)	48.0 (44.2-55.0)	0.801

Age is representative of age at the time of surgery; BMI: Body Mass Index; CVD: Cardiovascular Disease; HDL-C: High Density Lipoprotein Cholesterol; ApoA1: Apolipoprotein A1; LDL-C: Low Density Lipoprotein Cholesterol; hsCRP: highly sensitive C-reactive Protein; HbA1c: glycated haemoglobin; HOMA-IR: Homeostatic Model of Assessment for Insulin Resistance.

Anti-apoA-1 IgG associations with specific biological and clinical characteristics at baseline and after surgery

In the entire cohort at baseline (n=55), there was a significant correlation between hsCRP levels and anti-apoA-1 IgG levels (r=0.27; p=0.048), as well as inversely between serum apoA-1 concentration and anti-apoA-1 IgG levels (r=-0.30; p=0.028) (Table 3).There were no other significant correlations, including between the *percentage change* in measured variables and *percentage changes* in anti-apoA-1 IgG levels.

Similarly, apart from significant differences in the anti-apoA-1 IgG levels, there were no significant differences in the variables' percentage change or values at 12 months between seropositive and seronegative patients for anti-apoA-1 IgG.

A sub-analysis comparing the 8 patients whose post-operative antibody status remained positive with the 6 patients who became negative for anti-apoA-1 IgG showed, as expected, a significant reduction in anti-apoA-1 IgG levels in the subgroup whose antibody status changed to negative compared to those who remained positive (Figure 2). Although there was a trend for changes in other measures, none of these reached statistical significance (Figure 2). There were no significant correlations between anti-apoA-1 IgG levels and other variables seen in either group (positive at baseline who stayed positive and those who changed status to negative) at baseline and six and twelve months post-operatively. Similarly, there were no significant correlations between the *percentage chan*ges in variables in either sub-group. The 2 patients with a history of CVD and anti-apoA-1 antibody positivity both changed their status to negative following bariatric surgery.

Table 3. Correlation co-efficient (r) between anti-apoA-1 IgG and other variables at different time-points

Baseline vs Anti-ApoA-1 IgG		**6 months post-operative vs Anti-ApoA-1 IgG**		**12 months post-operative vs Anti-ApoA-1 IgG**	
Total Cholesterol (mmol/L)	r = -0.07 p = 0.59	**Total Cholesterol (mmol/L)**	r = -0.04 p = 0.82	**Total Cholesterol (mmol/L)**	r = -0.01 p = 0.99
Triglycerides (mmol/L)	r = -0.149 p = 0.28	**Triglycerides (mmol/L)**	r = 0.08 p = 0.65	**Triglycerides (mmol/L)**	r = -0.08 p = 0.60
HDL-C (mmol/L)	r = -0.09 p = 0.51	**HDL-C (mmol/L)**	r = -0.12 p = 0.48	**HDL-C (mmol/L)**	r = 0.002 p = 0.99
ApoA1 (g/L)	**r = -0.344** **p = 0.01**	**ApoA1 (g/L)**	r = -0.27 p = 0.11	**ApoA1 (g/L)**	r = -0.12 p = 0.39
LDL-C (mmol/L)	r = -0.04 p = 0.80	**LDL-C (mmol/L)**	r = 0.001 p = 0.99	**LDL-C (mmol/L)**	r = 0.008 p = 0.95
hsCRP (mg/L)	**r = 0.272** **p = 0.04**	**hsCRP (mg/L)**	r = 0.09 p = 0.60	**hsCRP (mg/L)**	r = 0.19 p = 0.18
HbA1c (mmol/mol)	r = 0.137 p = 0.32	**HbA1c (mmol/mol)**	r = 0.09 p = 0.62	**HbA1c (mmol/mol)**	r = 0.07 p = 0.63
Fasting Glucose (mmol/L)	r = -0.08 p = 0.56	**Fasting Glucose (mmol/L)**	r = 0.20 p = 0.27	**Fasting Glucose (mmol/L)**	r = -0.003 p = 0.98
HOMA-IR	r = -0.09 p = 0.51	**HOMA-IR**	r = 0.20 p = 0.27	**HOMA-IR**	r = 0.001 p = 0.99
BMI (kg/m^2)	r = 0.107 p = 0.44	**BMI (kg/m^2)**	r = 0.22 p = 0.20	**BMI (kg/m^2)**	r = 0.12 p = 0.38

Spearman's correlation co-efficient between different variables at different time points. Anti-apoA-1 IgG: antibodies to apolipoprotein A-1; HDL-C: High-density lipoprotein cholesterol; ApoA1: apolipoprotein A-1; LDL-C: low-density lipoprotein cholesterol; hsCRP: high-sensitivity C-reactive protein; HbA1c: glycated haemoglobin; HOMA-IR: homeostatic model of assessment of insulin resistance; BMI: body mass index.

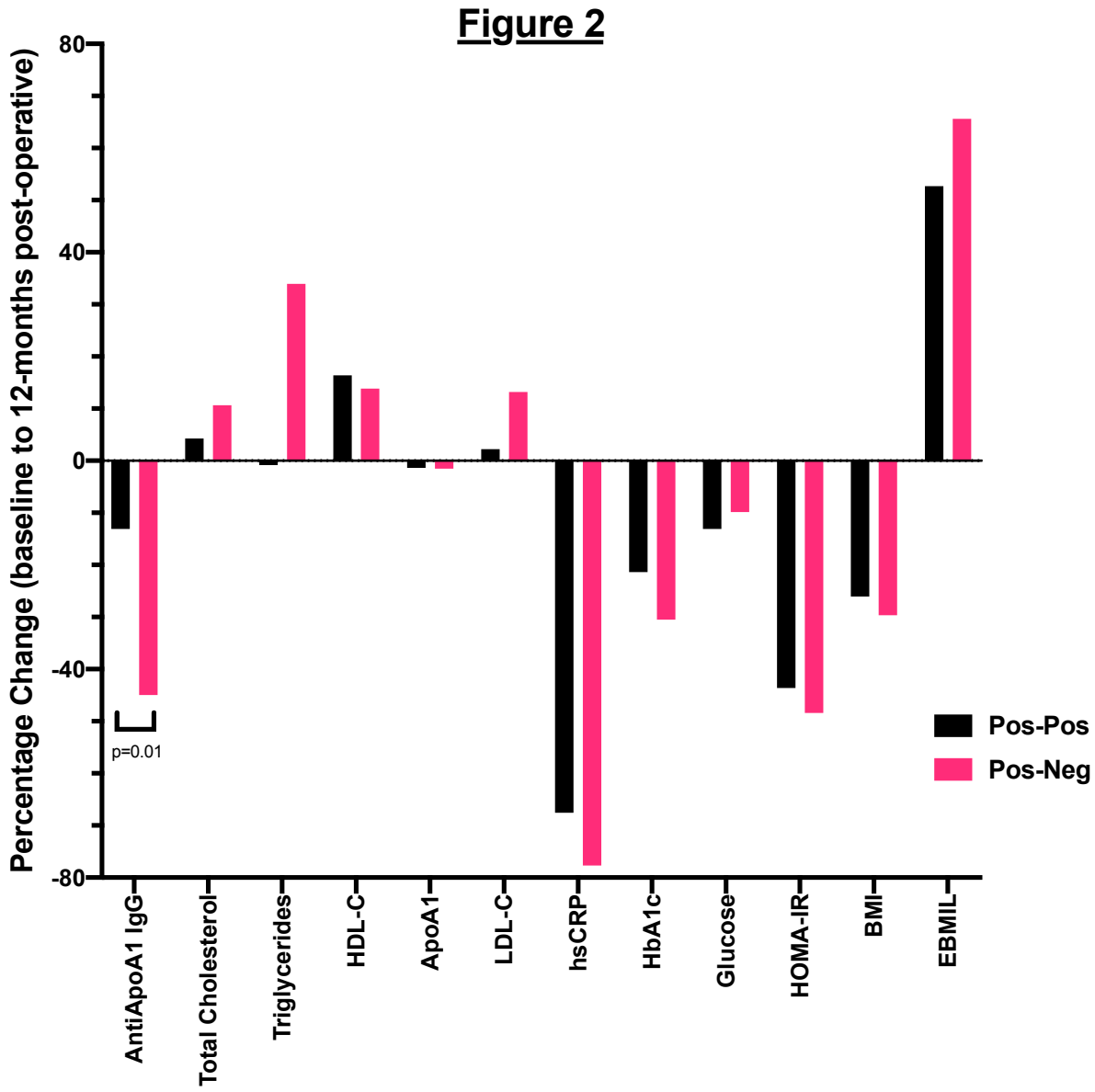

Figure 2. Differences in variables between patients whose antibody status changed from positive to negative compared to those whose antibody status remained positive.

There were no statistically significant differences between groups (apart from antibody levels) but there was a trend for greater reductions in hsCRP, HOMA-IR, BMI and increases in %EBMIL, HDL-C and triglycerides. AntiApoA1: antibodies to apolipoprotein A1; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; hsCRP: high-sensitivity C-reactive protein; HOMA-IR: homesostatic model of assessment of insulin resistance; BMI: body mass index; EBMIL: percentage excess BMI loss above 25 kg/m^2.

Anti-apoA-1 IgG levels and the relationship with 1-year post-surgery percentage of Excess Body Mass Index Loss (%EBMIL)

Patients whose antibody status at 12-months post-surgery was negative (n=47) displayed a significantly (p=0.03) greater mean %EBMIL (68%) when compared to those patients (n=8) whose 12-month post-operative antibody status was positive (mean %EBMIL of 53%) (Figure 3). There were no other significant differences in variables between those who were positive compared to those whose antibody status was negative at 12-months post-bariatric surgery. Logistic regression analysis showed that the 12-month post-operative anti-apoA-1 IgG levels independently exerted an adverse effect on the achievement of a %EBMIL of >50% (Table 4).

Figure 3: 12 Month Comparison of %EBMIL

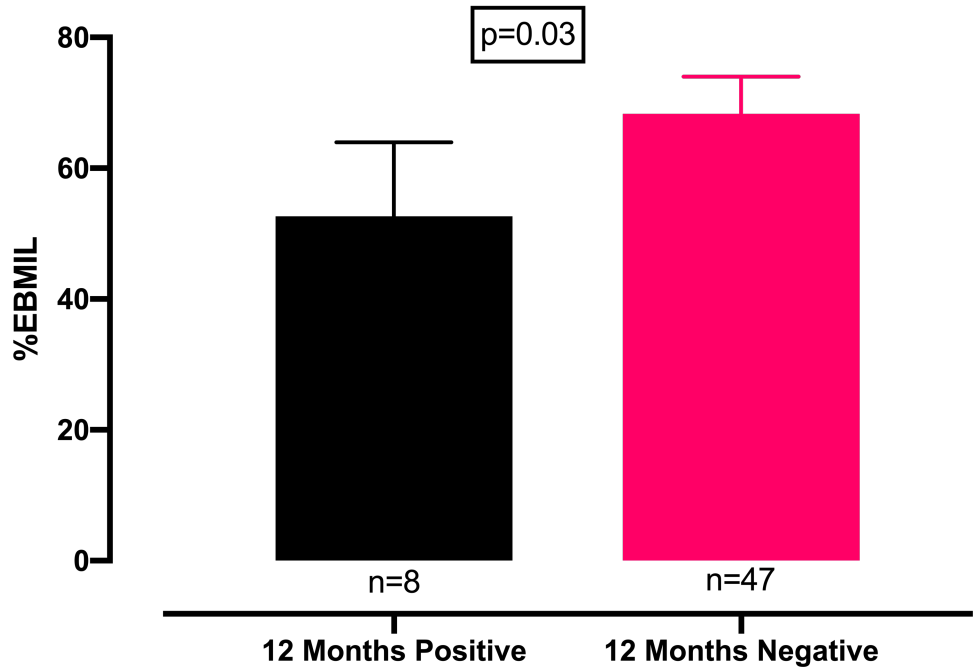

Figure 3: Comparison of %EBMIL in patients whose antibody status was positive or negative twelve months after bariatric surgery.

There was a statistically significant difference in the %EBMIL achieved in those with negative antibody titres. %EBMIL: percentage of excess body mass index loss above 25 kg/m^2

Table 4: Binary regression model assessing factors predicting %EBMIL >50%

Dependent variable: %EBMIL >50%
R^2 = 0.42; p=0.01

Co-variates	*Odds Ratio*	*p*
Baseline BMI (kg/m^2)	0.912	0.134
Baseline anti-apoA-1 IgG	3.26	0.404
12-month post-operative anti-apoA-1 IgG	0.003	**0.029**
Age	0.833	**0.007**
Procedure	3.17	0.170

A binomial logistic regression was performed to ascertain the effects of age, baseline BMI, twelve-month post-operative anti-apoA-1 IgG levels and procedure type on the likelihood that participants had an EBMIL greater than 50% (as a categorical variable). Of the five predictor variables only two were statistically significant: age and twelve-month post-operative anti-apoA-1 IgG levels.
BMI: Body Mass Index; anti-apoA-1 IgG: antibodies to apolipoprotein A-1.

Discussion

The novel findings of this study indicate that bariatric surgery reduces anti-apoA-1 IgG levels in severely obese patients as early as 6 months post-operatively, and that post-operative anti-apoA-1 IgG levels were significantly associated with a decreased EBMIL at 1-year, suggesting that the sustained presence of these antibodies might negatively influence weight-loss. Indeed, on regression analysis, the 12-month post-operative anti-apoA-1 IgG levels were shown to independently affect the achievement of an %EBMIL of >50%. We did not find any relationships or predictive factors between changes in anti-apoA-1 IgG levels and other biochemical variables.

Interestingly, the prevalence of autoantibody positivity status at baseline in our cohort (25%) was greater than the general population (n=6649) prevalence found in the CoLaus study (19.9%) which comprised of a community with a relatively high CVD prevalence (12, 31). It was also higher than the prevalence in a cohort of patients who were on dialysis for end-stage renal failure, another high-CVD risk group (32). Furthermore, the baseline anti-apoA-1 autoantibody positivity prevalence in our cohort was almost 2-fold higher than a cohort of severely obese patients without any history of any metabolic complications (21). However, after bariatric surgery, the prevalence rates in anti-apoA-1 antibody status was similar in our cohort to the “metabolically healthy” obese patients (21). This indicates a significant role of obesity as a cardiometabolic risk, mediated via compromising HDL properties. We also show, in keeping with our previous studies, marked improvements in BMI, insulin resistance, hyperglycaemia, inflammation and the lipid profile (25, 33-35).

Obesity has been shown to be an independent risk factor for CVD and the occurrence of traditional risk factors (chronic hyperglycaemia, hypertension and dyslipidaemia) is higher in obese persons compared to non-obese persons (36). Additionally, non-traditional risk factors such as inflammation have also been shown to be higher in obese patients such that obesity has been regarded as a chronic inflammatory condition (37). Importantly, in a previous study done in the setting of another chronic inflammatory condition, rheumatoid arthritis, anti-apoA-1 antibodies were found to be an independent predictor of the presence of CVD (18). Indeed, in

this study, hsCRP levels were higher in the patients whose antibody status was positive compared to negative. Similarly, in our study, there was a moderate correlation between hsCRP levels and anti-apoA-1 IgG levels. Other studies have shown associations between anti-apoA-1 IgG levels, CRP and inflammatory cytokines (12, 13, 16, 38) .

Anti-apoA-1 antibodies were previously found to also be independent predictors of all -cause mortality (11) and incident CVD in the general population (39). Moreover, in otherwise healthy obese persons, anti-apoA-1 antibodies were predictive of coronary artery calcification (21). Although the numbers were small, in our study, there was a greater proportion of patients with CVD in the patients who tested positive at baseline compared to those who tested negative for anti-apoA-1 antibodies (Table 2). This observation may further underline the role that anti-apoA-1 antibodies have in the pathophysiology of atherosclerotic CVD in obesity and the reduction of these antibodies may be a mechanism by which CVD is reduced post-bariatric surgery.

Although we did not find any relationships between baseline anti-apoA-1 IgG levels and autoantibody positivity status with either weight or BMI, we did observe a greater %EBMIL in patients who were negative compared to those who were positive post-operatively. This may indicate that the ongoing presence of these antibodies might negatively influence weight-loss. Our sample size is relatively small thereby restricting any definitive conclusions and this observation will need further validation and elucidation in larger clinical studies.

We demonstrated, at baseline, an inverse correlation between anti-apoA-1 IgG levels and apoA-1 levels. This trend has been observed in a previous study examining anti-apoA-1 in type 2 diabetes (40) and although our study was not exclusively in patients with type 2 diabetes, more than half the participants had a history of it. Another recent study in patients with hepatitis C showed that patients who displayed anti-apoA-1 positivity also had significantly lower apoA-1 levels (41). It has been established that obesity itself alters HDL metabolism (particularly apoA-1) (42) and it may be that the presence of anti-apoA-1 antibodies augments this derangement and therefore enhances CVD risk.

In our analysis we could not find predictive factors responsible for the reduction in anti-apoA-1 antibody levels. Bariatric surgery improves known (e.g. inflammation, dyslipidaemia, hyperglycaemia (33)) systemic CVD risk factors concurrently. In the aforementioned previous study on patients with hepatitis C, treatment for the virus did not provide as marked a reduction in either the anti-apoA-1 titres nor in positivity status (41). Interestingly, a large (n=499) longitudinal (mean follow-up period 12.1 years) British study in patients with SLE showed that early positivity of Anti-apoA-1 antibodies did not affect either survival or CVD incidence (43). The study shows a fluctuance of anti-apoA-1 antibody levels with disease activity and that patients on hydroxychloroquine therapy displayed 30% lower levels. The authors did not report whether a change in antibody status over time (e.g. going from positive to negative at times of therapy/reduction in disease activity) may have impacted on the observations relating to CVD and mortality outcomes (43). It is therefore unclear whether over time, changing from a positive to a negative status can reduce CVD risk. A further longitudinal interventional study in obese persons with both surgical and non-surgical weight-loss may help to clarify matters further.

We acknowledge certain limitations in this study. Firstly, the sample size, although allowing us to satisfy the primary aim of the study, did not allow us to perform a detailed analysis of the factors driving changes in anti-apoA-1 antibodies post-bariatric surgery. We also had a greater proportion of women which did not allow us to elucidate sex-specific differences. Furthermore, due to the relatively small numbers, we could not definitively assess for potential procedure related differences. Finally, the prevalence of CVD in our cohort was low and duration of follow-up was relatively short to determine whether the post-bariatric surgery reductions in anti-apoA-1 antibodies have any translatable effect on CVD outcomes.

In summary, we show for the first time that bariatric surgery, through unknown (but likely multifactorial) mechanisms, reduces anti-apoA-1 antibody levels and changes positivity status. Whether this in part explains the reduction in CVD seen in longitudinal cohort studies in patients following bariatric surgery requires further study. The observation that patients whose antibody status was negative had a greater %EBMIL requires needs to be explored further.

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Author Contributions

S.A. conducted a literature search, collected data, analysed and interpreted data and wrote the first draft of the manuscript. J.H. reviewed relevant literature, collected data and worked on the first draft of the manuscript. Y.L, T.S., S.P., S.A., S.D. collected data and reviewed the manuscript. R.D., B.A. and A.S collected data, interpreted data and reviewed the manuscript. N.V and H.S. designed the study, interpreted data and reviewed the manuscript. All authors have critically reviewed the final version of the manuscript.

Declaration of Interests

H.S. has received grants and personal fees from Akcea, grants and personal fees from Amgen, grants from Pfizer, grants and personal fees from MSD, personal fees from Sanofi, personal fees from Synageva, personal fees from Takeda, which are all outside of the submitted work. R.M has received grants and personal fees from Pfizer and Novo Nordisk. None of the other authors have any relevant conflicts of interest to declare.

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Chapter 7: Improvements in Diabetic Neuropathy and Nephropathy after Bariatric Surgery: A Prospective Cohort Study

Currently under review.

Author's Contribution: Safwaan Adam researched data (patient recruitment, clinical assessments, venous blood sample collection, and serum and plasma separation for storage), analysed and interpreted data. In addition, Safwaan Adam researched the available literature and wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript. The manuscript formed the basis of this chapter.

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Improvements in Diabetic Neuropathy and Nephropathy after Bariatric Surgery: A Prospective Cohort Study

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Tables: 4

Figures: 2 (and 1 supplementary)

Abstract

Background:

There are limited data on the impact of bariatric surgery on microvascular complications of type 2 diabetes (T2D), particularly diabetic neuropathy. We assessed microvascular complications (especially neuropathy) in obese patients with T2D before and 12-months after bariatric surgery.

Methods:

This was a prospective observational cohort study at a bariatric referral centre. Twenty-six (62% female; median age 52 years) obese patients with T2D were recruited. Measurements of neuropathy symptom profile (NSP), neuropathy disability score (NDS), quantitative sensory testing (QST), vibration perception threshold (VPT), nerve conduction studies (NCS) and corneal confocal microscopy (CCM) to quantify corneal nerve fibre density (CNFD), branch density (CNBD) and fibre length (CNFL); urinary albumin:creatinine ratio (uACR), estimated glomerular filtration rate (eGFRcyst-creat) and retinal grading.

Results:

Body mass index (BMI) (47.2 to 34.5 kg/m2; $p<0.001$) decreased post-operatively. There were improvements in CNFD (27.1 to 29.2/mm2; $p=0.005$), CNBD (63.4 to 77.8/mm2; $p=0.008$), CNFL (20.0 to 20.2/ mm2; $p=0.001$), NSP (3 to 0/38; $p<0.001$) and eGFRcyst-creat (128 to 120 ml/min; $p=0.015$) after bariatric surgery. Changes in (Δ) triglycerides were associated with ΔCNFL ($p<0.05$) and Δsystolic blood pressure (SBP) and excess BMI loss were associated with ΔeGFRcyst-creat. There was no significant change in NDS, QST, VPT, NCS, uACR or retinopathy status. Glomerular hyperfiltration resolved in 42% of the 12 patients with this condition pre-operatively.

Conclusion:

Bariatric surgery results in improvements in small nerve fibres and glomerular hyperfiltration in obese people with T2D, which were associated with weight loss, triglycerides and SBP, but with no change in retinopathy or uACR at 12-months.

Introduction

Obesity is a major contributor to the epidemic of type 2 diabetes and much of the health and economic burden of type 2 diabetes relates to its microvascular and macrovascular complications. Bariatric surgery is an effective and durable treatment for the remission of type 2 diabetes (1) and has long-term benefits for incident major macrovascular and microvascular events. However, there are few detailed studies assessing early outcomes in relation to microvascular complications, particularly neuropathy.

A previous health-record based retrospective cohort study showed that remission of type 2 diabetes after bariatric surgery conferred protection against the development of microvascular complications even after relapse of type 2 diabetes (2). Similarly, in a large retrospective cohort study, 5-year incident microvascular complications were reduced by 78% after bariatric surgery. Because microvascular complications were defined by the end stage outcomes of amputation, laser eye surgery or blindness and dialysis (3), the benefits of bariatric surgery have not been reported until at least 5 years of follow up (4). Likewise, the longitudinal Swedish Obese Subjects (SOS) study showed a significant reduction in microvascular outcomes, 15 years after bariatric surgery, as they were evaluated using crude disease codes for retinopathy, nephropathy and neuropathy from the National Patient Register (5).

Correspondingly, a recent retrospective study using healthcare record diagnostic codes also showed a reduction in the incidence of microvascular complications in subjects with type 2 diabetes after a median follow-up of 4.3 years following bariatric surgery (6).

More detailed short-term studies of microvascular outcomes have however reported conflicting results. A recent meta-analysis of seven controlled studies found that bariatric surgery prevented the development of diabetic retinopathy but did not impact on progression or regression of retinopathy (7). With regard to nephropathy, urinary albumin:creatinine ratio (uACR) was reduced and both glomerular hypo- and hyperfiltration improved after bariatric surgery (8, 9). Data relating to diabetic neuropathy are contradictory, as there are reports of worsening symptomatic neuropathy, attributed to nutritional deficiencies (10) and a 33% incidence of neuropathic pain following bariatric surgery (11). In a prospective cohort study of 20 participants undergoing bariatric surgery, there were significant improvements in the

neuropathy symptom and disability scores after 6 months (12). However, Miras *et al.* found no significant improvement in radial, sural and peroneal nerve conduction velocities or amplitudes 12 months after bariatric surgery (13).
We have assessed the effect of bariatric surgery on microvascular complications in a cohort of obese patients with type 2 diabetes undergoing bariatric surgery. We have undertaken detailed assessment of small fibre neuropathy using corneal confocal microscopy (CCM) as it showed early small fibre repair in our previous study following simultaneous pancreas and kidney transplantation (SPK) in T1DM (14).

Methods

Study Design and Patient Recruitment

We prospectively studied 26 obese patients with type 2 diabetes undergoing bariatric surgery at Salford Royal Hospital, a Tier 4 specialist weight management service in the North West of England. Assessments were undertaken before and 12 months after bariatric surgery. Participants with a history of corneal trauma, surgery or disease were excluded from the study. Patients with a history of retinal, renal or neuropathic disease not due to type 2 diabetes were also excluded.
Ethical approval was sought and granted by the Central Manchester Research and Ethics Committee with all patients providing informed consent before study participation.

Clinical and Laboratory Assessment

Body mass index (BMI) was assessed at each visit and the per cent excess BMI loss (%EBMIL) was calculated using the difference in proportionate change in BMI in excess of the upper limit of normal BMI of 24.9 kg/m^2 before and after bariatric surgery. Blood pressure was measured after resting in a seated position for 5 minutes, using an Omron HEM 705-CP semiautomatic oscillometric recorder. Fasting venous blood samples and early morning urine samples were collected at each visit. Glycated hemoglobin (HbA1c), serum creatinine, uACR, total cholesterol, triglycerides and high density lipoprotein cholesterol (HDL-C) were measured in the biochemistry laboratory at Manchester University Hospitals NHS Foundation Trust using routine methods. Serum cystatin C was assayed in the Cardiovascular Research Group Lab at the University of Manchester using immunoturbidimetric

assays with a Cobas Mira analyser (Horiba ABX Diagnostics, Nottingham, UK). The laboratories participated in the UK National External Quality Assessment Service (UKNEQAS, Birmingham, UK) for quality control of general blood chemistry and urinary chemistry. Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula.
Complete remission from type 2 diabetes was classified with an HbA1c below 6.0 % (42 mmol/mmol) and no active pharmacological therapy, as per the American Diabetes Association consensus statement (15).

Diabetic Neuropathy

The neuropathy symptom profile (NSP) questionnaire consists of 38 questions which assess symptoms of sensory, motor and autonomic neuropathy. The neuropathy disability score (NDS) includes an assessment of pinprick, temperature sensation (using hot and cold rods), vibration sensation (tuning fork) and ankle reflexes. A score between 0 and 2 is considered normal, 3-5 is mild neuropathy, 6-8 is moderate neuropathy and 9-10 is severe neuropathy. A Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK) was used to determine the vibration perception threshold. Cold and warm thermal thresholds were determined using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel) on the S1 dermatome of the left foot. Nerve conduction studies (NCS) were performed by a consultant neurophysiologist using a “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to maintain limb temperature between 32-35°C. Deep breathing heart rate variability was measured using an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies, Philadelphia, PA, USA).

Corneal Confocal Microscopy

Corneal confocal microscopy (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) comprising six non-overlapping corneal images per patient (three per eye) from the centre of the cornea was performed using our established protocol (16). Three corneal nerve parameters were quantified manually quantified using CCMetrics (The University of Manchester, Manchester, UK): corneal nerve fibre density (CNFD) - the total number of major

nerves/mm^2 of corneal tissue, corneal nerve branch density (CNBD) - the number of branches emanating from the major nerve trunks/mm^2 of corneal tissue and corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue.

Diabetic Kidney Disease

The CKD-EPI (2012) equation (combining cystatin C and creatinine, unadjusted for body surface area; CKD-EPIcyst-creat) was used to determine estimated glomerular filtration rate (eGFR). Glomerular hyperfiltration was defined as an eGFR >125 ml/min in keeping with a recent meta-analysis by Li *et al.* which analysed changes in eGFR post bariatric surgery (17). Albuminuria was defined as uACR >3.5 mg/mmol in women and >2.5mg/mmol in men.

Diabetic Retinopathy

Two (optic disc and macula centred) 45-degree digital retinal images were used to grade retinopathy from the NHS Diabetic Eye Screening Programme (NHS DESP), as part of the patient's routine diabetes clinical care before and after surgery. Nationally accredited screeners classified diabetic retinopathy status according to the NHS DESP Feature Based Grading Classification (18). For quality assurance, an independent ophthalmologist also graded images without prior knowledge of the pre-existing grading.

Statistical Analysis

SPSS for Mac (Version 23.0, IBM SPSS Statistics, Armonk, NY: IBM Corp.) and GraphPad Prism (Version 7.00, GraphPad Software, La Jolla California USA) were used for analysis of data. Tests for normality were done using the Shapiro-Wilk test, visualisation of histograms and Q-Q plots. To compare means pre- and post-bariatric surgery, paired t-tests were used for normally distributed variables; Wilcoxon matched pairs test was used for non-parametric variables and McNemar's test for categorical variables. Tests for relationships between percentage changes (from baseline to 12 months) in variables utilised Pearson's co-efficient for parametric data and Spearman's co-efficient for non-parametric data. Multifactorial linear regression was used to assess for associations between percentage changes in variables. Variables chosen for regression models were based on predicted influential factors.

A p-value of <0.05 was considered as statistically significant. The *a priori* estimated sample size required to assess for changes in CCM parameters (the main outcome of interest) was 23 patients to achieve an alpha of 0.05 and statistical power of 80%. These calculations were based on pilot data obtained for a different project in a similar cohort (unpublished data on file).

Results

Participant Characteristics

We assessed 26 participants at baseline and 12 months after bariatric surgery (gastric bypass (n=21), sleeve gastrectomy (n=5) (Table 1).

There was a significant reduction in BMI ($p<0.001$), with a mean %EBMIL of 61±16 %. Complete remission of type 2 diabetes occurred in 21 out of 26 (77%) participants ($p=0.0001$). There were significant reductions in patients using insulin ($p=0.04$), angiotensin converting enzyme inhibitors or angiotensin II receptor blockers (ACEi/ARB) ($p=0.02$) and statins ($p=0.04$) (Table 1). There were significant reductions in systolic ($p<0.001$) and diastolic ($p<0.02$) blood pressures and HbA1c ($p<0.001$) and an increase in total cholesterol ($p=0.04$) and HDL ($p<0.001$) (Table 1).

Table 1. Clinical and metabolic variables pre and post-bariatric surgery.

Variable (n=26)	Baseline	12 Months	p-value
Age	52 (10)		
Female (%)	16 (62%)		
Diabetes Duration (years)	6 (3-12)		
Insulin Treatment	8 (31%)	2 (8%)	**0.041**
ACE-I or ARB Treatment	18 (69%)	11 (42%)	**0.023**
Statin Treatment	19 (73%)	13 (50%)	**0.041**
Weight	137 (120-152)	93 (85-117)	**<0.001**
BMI (kg/m^2)	47.2 (43.0-57.0)	34.5 (30.0-38.4)	**<0.001**
Systolic BP (mmHg)	134 (15)	119 (15)	**<0.001**
Diastolic BP (mmHg)	75 (73)	70 (11)	**0.016**
HbA1c (%)	6.9 (6.4-8.6)	5.5 (5.3-6.0)	**<0.001**
(mmol/mol)	52 (46-71)	37 (34-42)	
Total Cholesterol (mg/dL)	144 (28.6)	162 (36.7)	**0.035**
(mmol/l)	3.72 (0.74)	4.20 (0.95)	
Triglycerides (mg/dl)	134 (81.4-165)	100 (77.0-132)	0.071
(mmol/l)	1.51 (0.92-1.86)	1.13 (0.87-1.49)	
HDL-C (mg/dl)	33.2 (29.7-39.0)	44.0 (38.6-50.6)	**<0.001**
(mmol/l)	0.86 (0.77-1.01)	1.14 (1.00-1.31)	
LDL-C (mg/dl)	81.9 (23.9)	93.8 (35.1)	0.198
(mmol/l)	2.12 (0.62)	2.43 (0.91)	

Data presented as mean (SD) or median (interquartile range). There were significant ($p<0.05$) reductions in the use of medication after surgery. Weight, BMI, blood pressure, HbA1c, total cholesterol, triglyceride and HDL cholesterol improved post-operatively.
ACE-I: Angiotensin Converting Enzyme Inhibitors; A2RB: Angiotensin II Receptor Blockers; BMI: Body Mass Index; BP: Blood Pressure; HbA1c: Glycated Haemoglobin; HDL-C: High Density Lipoprotein Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol.

Diabetic Neuropathy

Neuropathy symptoms (NSP) showed a significant improvement (p<0.001). Based on the NDS, 6/26 participants had DPN at baseline (4 mild, 1 moderate, 1 severe) and showed a non-significant (p=0.07) trend for improvement after bariatric surgery (Table 2). Quantitative sensory testing showed no significant improvements in vibration, cold or warm perception thresholds 12 months after bariatric surgery (Table 2).

Table 2. Microvascular assessments pre and post-bariatric surgery.

Parameter	Baseline	12 Months	p
Neuropathy Assessment			
NSP (x/38)	3 (0-5)	0 (0-1)	**<0.001**
NDS (x/10)	1 (0-3)	0 (0-2)	0.068
VPT (volts)	14.2 (7.06)	13.6 (7.11)	0.969
CT Perception (°C)	25.7 (20.0-28.1)	26.3 (22.0-28.3)	0.702
WT Perception (°C)	40.0 (3.98)	41.3 (4.76)	0.093
DB-HRV (beats/min)	15 (12-22)	14 (11-20)	0.670
CNFD (no./mm^2)	27.1 (20.8-30.2)	29.2 (25-34.9)	**0.005**
CNBD (no./mm^2)	63.4 (35.1)	77.8 (35.5)	**0.008**
CNFL (mm/mm^2)	20.0 (15.8-22.7)	20.2 (18.3-23.8)	**0.001**
Renal Assessment			
uACR (mg/mmol)	1.00 (0.57-1.71)	0.50 (0.34-1.00)	0.103
sCreat (µmol/l) **(mg/dl)**	77 (27) 0.87 (0.31)	66 (17) 0.75 (0.19)	**<0.001**
sCysC (mg/l)	0.9 (0.72-1.03)	0.87 (0.79-1.11)	0.348
eGFR (ml/min)	128 (26)	120 (23)	**0.015**

Data are presented as mean (SD) or median (interquartile range).
There was a significant improvement in the NSP, CNFD, CNBD and CNFL (p<0.01). Other variables showed a non-significant trend towards improvement.
CNFD: Corneal Nerve Fibre Density; CNBD: Corneal Nerve Branch Density; CNFL: Corneal Nerve Fibre Length; NSP: Neuropathy Symptom Profile; NDS: Neuropathy Disability Score; VPT: Vibration Perception Threshold; CT: Cold Temperature; WT: Warm Temperature; DB-HRV: Deep Breathing Heart Rate Variability; ACR: albumin:creatinine ratio; sCreat: serum creatinine; sCysC: serum cystatin C; eGFR: estimated glomerular filtration rate.

Corneal Confocal Microscopy

Corneal nerve fibre density (p<0.005), nerve branch density (p=0.008) and nerve fibre length (p=0.001) improved significantly after bariatric surgery (Table 2, Figures 1 and 2).

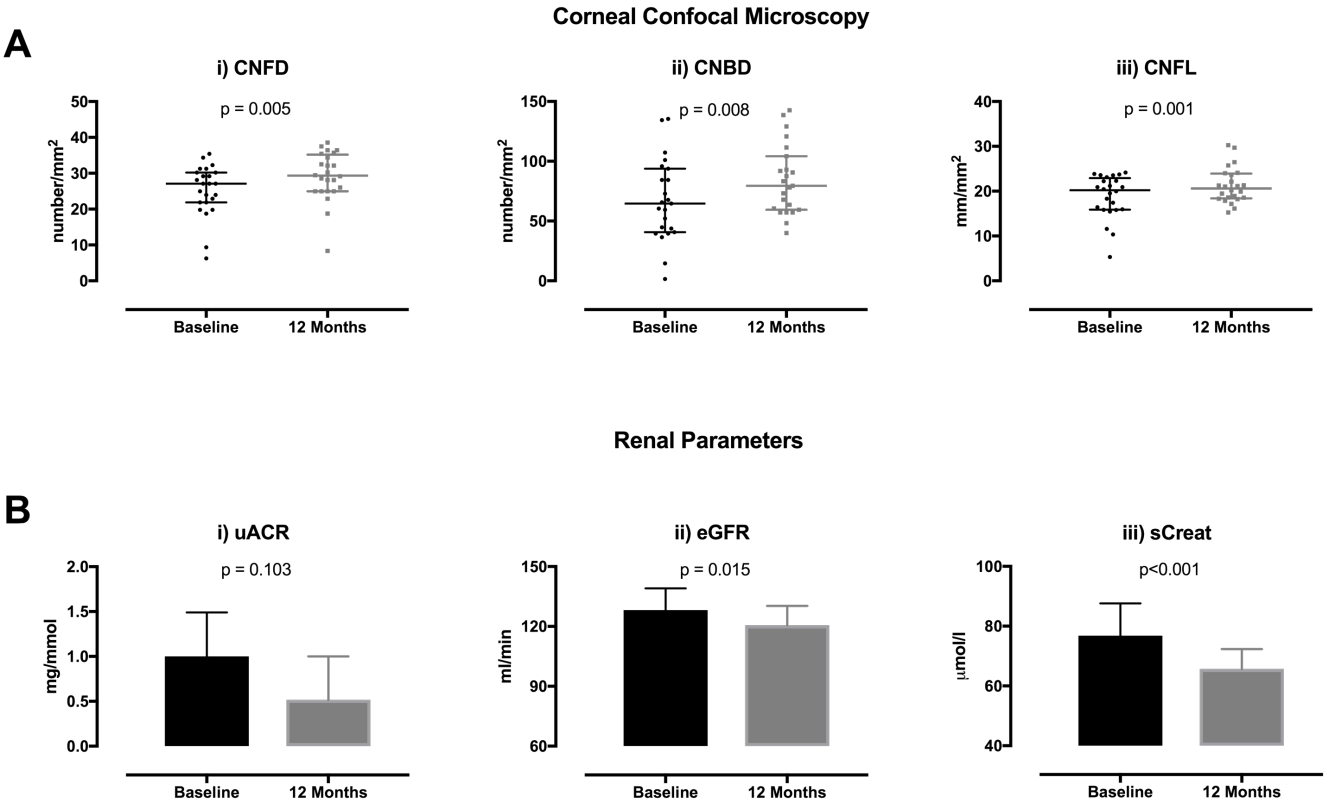


Figure 1. Microvascular outcome parameters before and after bariatric surgery.

Figure 1 depicts significant improvements in the CNFD, CNBD and CNFL from baseline to 12 months after bariatric surgery. There were also significant reductions in the eGFR, serum creatinine and a change in uACR (non-significant).
A *Corneal confocal microscopy*
A (i) Corneal Nerve Fibre Density before and after surgery
(ii) Corneal Nerve Branch Density before and after surgery
(iii) Corneal Nerve Fibre Length before and after surgery
B *Renal Parameters*
B (i) Urinary Albumin:Creatinine Ratio before and after surgery
(ii) Estimated Glomerular Filtration Rate before and after surgery
(iii) Serum Creatinine before and after surgery.

CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density; CNFL: corneal nerve fibre length; uACR: urinary albumin:creatinine ration; eGFR: estimated glomerular filtration rate; sCreat: serum creatinine.

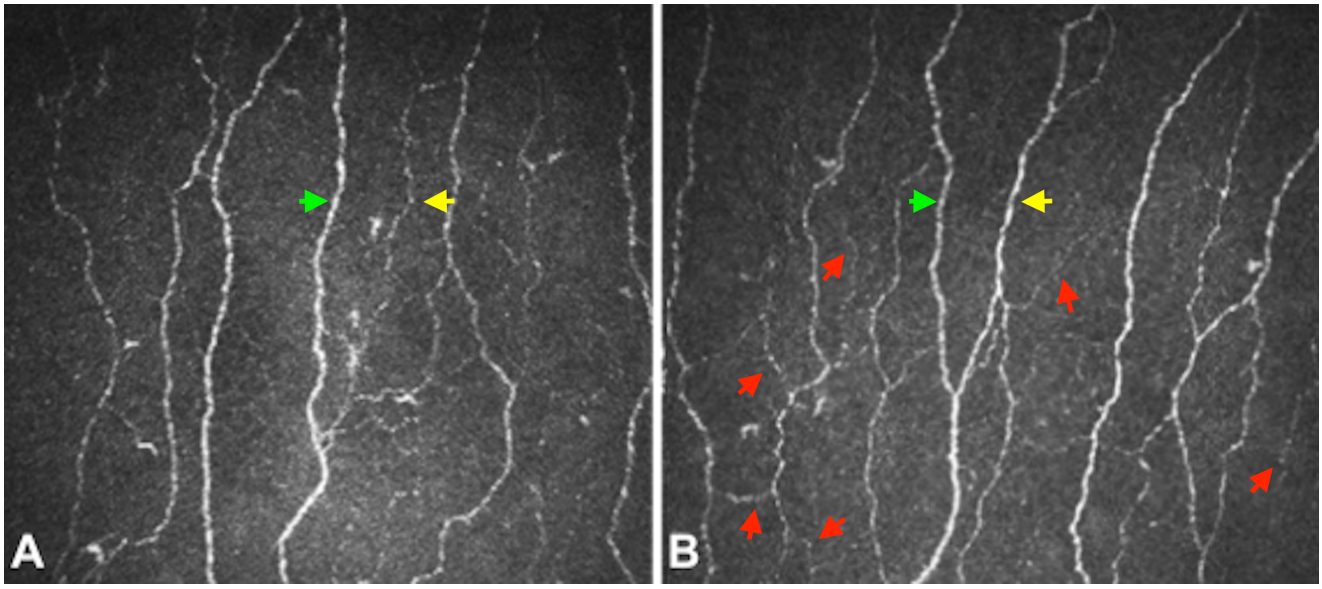


Figure 2. Example corneal confocal microscopy image in a participant

This image obtained using corneal confocal microscopy shows an improvement in the corneal nerve morphology from pre- (A) to post (B) bariatric surgery. In the post-operative image (B) there are more nerves seen and the red arrows depict small nerve fibre branches indicative of regeneration. The green arrows illustrate a main nerve fibre and the yellow arrows depict nerve fibre branches.

Cardiac Autonomic Function

There was no change in deep breathing heart rate variability after bariatric surgery (Table 2).

Nerve Conduction Studies

NCS was performed in 9/26 patients before and after surgery with no significant difference between participants who did and did not undergo NCS in relation to baseline BMI, blood pressure, diabetes duration and measures of neuropathy. There was no significant change in sural nerve latency, amplitude, conduction velocity, peroneal nerve latency, amplitude and velocity and radial nerve amplitude and velocity (Table 3).

Table 3. Nerve Conduction Studies pre and post-bariatric surgery.

Parameter (n=9)	Baseline	12 Months	p-value
Sural Latency (ms)	2.84 (0.55)	3.04 (0.32)	0.384
Sural Amplitude (μV)	8.15 (5.68)	6.94 (3.84)	0.820
Sural Velocity (m/s)	49.9 (6.69)	46.5 (4.04)	0.380
Peroneal Latency (ms)	4.07 (0.46)	4.23 (0.42)	0.221
Peroneal Amplitude (mV)	3.14 (0.94)	3.08 (1.35)	0.905
Peroneal Velocity (m/s)	45.6 (5.06)	45.3 (3.22)	0.848
Radial Amplitude (μV)	33.2 (11.5)	34.9 (12.3)	0.216
Radial Velocity (m/s)	61.4 (3.09)	59.4 (4.05)	0.275

Data are presented as mean (SD).

Diabetic Retinopathy

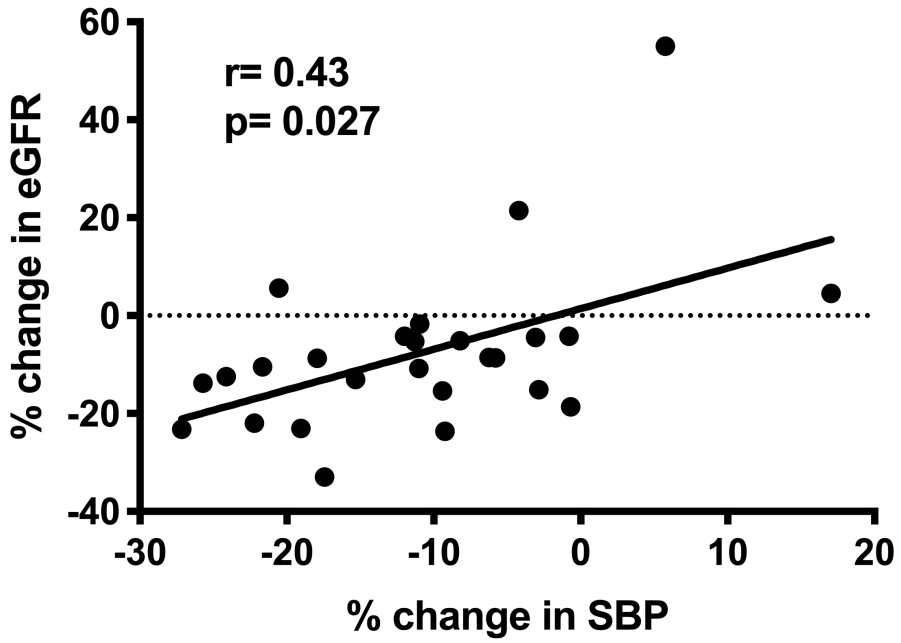
At baseline 5 (19%) participants had background retinopathy (R1); it improved in one patient with no deterioration in any of the participants. One patient had maculopathy at baseline, which had resolved at follow up without any specific treatment for maculopathy.

Diabetic Kidney Disease

There was a significant decrease in serum creatinine ($p<0.001$), no change in serum cystatin C (Table 2) and a significant decrease in eGFR ($p<0.02$) after bariatric surgery (Table 2; Figure 1). Pre-operatively 12 (46%) patients fulfilled the criteria for glomerular hyperfiltration and the eGFR fell below 125 ml/min in 5 participants after surgery ($p=0.07$). There was no significant decrease in uACR ($p=0.10$) (Table 2; Figure 1). Seven patients stopped ACEi therapy due to normalization of blood pressure. There were no significant differences in baseline or post-operative results between patients who were being treated with ACEi compared to those who were not.

Relationships between changes in metabolic parameters and microvascular outcomes

There was no correlation between the change in BMI, HbA1c and lipids and change in microvascular complications. Changes in SBP correlated with changes in eGFR (Supplementary Figure 1).



Supplementary Figure 1. Relationship between percentage change in estimated glomerular filtration rate and percentage change in systolic blood pressure

Changes in the percentage eGFR and SBP showed a significant correlation. eGFR: estimated glomerular filtration rate; SBP: systolic blood pressure.

Multifactorial regression was used to assess for the potential influence of changes in metabolic measures on microvascular disease outcomes (Table 4). There was a significant association between change (∆) in triglycerides (B= -0.53; p=0.024) and change in CNFL and %EBMIL (B= -0.004; p=0.018) and ∆SBP (B=0.62; p=0.017) with ∆eGFR.

Table 4. Association between bariatric surgery induced changes in metabolic variables and changes in microvascular outcome measures.

Variable	Co-efficient	95% Confidence Interval	p
Percentage Change in CNFL			
Pre-operative diabetes duration	0.002	-0.009 to 0.013	0.682
ΔHbA1c	0.158	-0.177 to 0.492	0.331
ΔSBP	0.497	-0.071 to 1.065	0.082
ΔTriglycerides	**-0.135**	**-0.250 to -0.020**	**0.024**
%EBMIL	0.004	0.000 to 0.008	0.05
Percentage Change in eGFR			
Pre-operative diabetes duration	0.002	-0.007 to 0.011	0.637
ΔHbA1c	-0.093	-0.383 to 0.196	0.502
ΔSBP	**0.619**	**0.127 to 1.111**	**0.017**
ΔTriglycerides	-0.066	-0.165 to 0.034	0.180
%EBMIL	**-0.004**	**0.001 to 0.007**	**0.018**
Percentage change in urinary ACR			
Pre-operative diabetes duration	**0.248**	**0.042 to 0.453**	**0.021**
ΔHbA1c	-4.108	-10.456 to 2.241	0.188
ΔSBP	-3.282	-14.069 to 7.505	0.526
ΔTriglycerides	-0.791	-2.971 to 1.390	0.452
%EBMIL	0.037	-0.034 to 0.108	0.286
Percentage change in CNFD			
Pre-operative diabetes duration	0.006	-0.082 to 0.094	0.884
ΔHbA1c	0.111	-2.643 to 2.864	0.932
ΔSBP	-1.047	-5.709 to 3.615	0.638
ΔTriglycerides	-0.204	-1.145 to 0.738	0.650
%EBMIL	0.020	-0.013 to 0.053	0.223
Percentage change in CNBD			
Pre-operative diabetes duration	-0.040	-0.093 to 0.012	0.121
ΔHbA1c	0.480	-0.955 to 1.914	0.485
ΔSBP	0.082	-2.375 to 2.539	0.944
ΔTriglycerides	-0.070	-0.599 to 0.459	0.782
%EBMIL	-0.001	-0.02 to 0.017	0.879

Multifactorial linear regression assessing relationships between percentage changes in microvascular outcomes and percentage change in metabolic variables. Diabetes duration relates to pre-operative duration of diabetes. Variables in bold text are statistically significant.
CNFL: corneal nerve fibre length; eGFR: estimated glomerular filtration rate; ACR: albumin:creatinine ratio; CNFD: corneal nerve fibre density; CNBD: corneal nerve branch density; %EBMIL: percentage excess body mass index loss (proportionate change in excess of BMI of >25 kg/m^2).
Δ = percentage change from baseline.

Discussion

This is the first study to show an improvement in diabetic neuropathy and diabetic kidney disease but not diabetic retinopathy, 12 months after bariatric surgery in obese patients with type 2 diabetes. There was a marked improvement in BMI, blood pressure, HbA1c and lipid profile, in keeping with previous studies (1) and a 77% remission rate of type 2 diabetes in our cohort.

Previous reports have shown a small fibre neuropathy (SFN) in obese patients without diabetes (19) and in participants with impaired glucose tolerance (20), particularly those who develop type 2 diabetes (21). Furthermore, a reduction in corneal nerve fibre length has been associated with age, HbA1c and HDL-C (22). The evaluation of small fibre damage was a major outcome in our study as CCM and skin biopsy have shown small nerve fibre damage before an abnormality in quantitative sensory testing and NCS in patients with sub-clinical diabetic neuropathy (23). We have also shown that corneal nerve fibre length is reduced in diabetic patients without microalbuminuria (24) or retinopathy (25) and predicts the development of diabetic neuropathy (26) and retinopathy (27). This indicates that CCM can detect early small fibre damage (28).

This study shows an improvement in NSP, which takes into account sensory, motor and autonomic symptoms, in keeping with the results of the DiaSurg1 study (12). The DiaSurg1 study also found an improvement in NDS, driven by changes in vibration perception and Achilles reflexes (12), implying large fibre benefits. However, in the present study, we found no improvement in NDS, vibration perception threshold or nerve conduction studies. This confirms the study by Miras *et al.*, which also found no significant improvement in radial, sural and peroneal nerve conduction velocities or amplitudes after bariatric surgery (13).
In the DiaSurg1 study there was no change in temperature perception or pinprick thresholds (12), suggesting no impact on small fibres. Whilst, we also found no improvement in cold and warm temperature thresholds or deep breathing heart-rate variability, there was a significant improvement in corneal nerve morphology. CCM has previously been used to show corneal nerve fibre repair, despite no change in neuropathy symptoms and deficits, neurophysiology, quantitative sensory testing

and skin biopsy in patients with T1DM following SPK (14, 29). A novel first-in-class peptide ARA290 (cibinetide) which blocks inflammation has been shown to improve corneal nerve fiber density and length in patients with sarcoidosis-related neuropathy (30, 31) and T2DM (32), which was paralleled by an improvement in pain scores and functional outcomes. In a 12-month trial of seal oil omega-3 polyunsaturated fatty acid in patients with T1DM there was a 29% increase in CNFL, with no change in nerve conduction velocity and sensory function (33).

Glomerular hyperfiltration may occur in the earliest phase of nephropathy in patients with type 2 diabetes. However, obesity *per se* is also associated with increased glomerular filtration. In our cohort, the mean preoperative eGFR was in keeping with glomerular hyperfiltration (17). Post-bariatric surgery, there is loss of adipose tissue, muscle mass and body surface area, which can impact upon the glomerular filtration rate. To account for this, our eGFR calculation utilised both changes in creatinine and cystatin C, and the eGFR measurement without indexing to body surface area (34). Our results show an apparent discordant relationship between serum creatinine and eGFR, as usually a fall in creatinine (as was seen post-operatively) should lead to a rise in eGFR. However, in our study eGFR was reduced after surgery as the calculation method we used accounted for changes in cystatin C and body surface area as opposed to creatinine alone. Indeed, Friedman *et al.* have shown that CKD-EPIcreat-cyst is the most accurate means of calculating eGFR against measured GFR in a bariatric cohort (34). The participants in this study on the whole had normal uACR readings and whilst there was a tendency towards a reduction in this parameter, this did not reach statistical significance. There was no change in retinopathy status in our cohort, which is in keeping with a recent meta-analysis (7).

In the current study, the reduction in triglycerides was associated with an increase in CNFL. Previous reports have shown that hypertriglyceridemia is a risk factor for the development of SFN in diabetic patients (35). Additionally, there was a direct relationship between the reduction in SBP and eGFR and the inverse relationship between excess BMI loss and ∆eGFR may indicate that weight loss reduces glomerular hyperfiltration, as both obesity and hypertension are risk factors for glomerular hyperfiltration. The change in HbA1c did not significantly influence change in any of the microvascular outcome measures, however, the baseline HbA1c was excellent and the change in HbA1c was small.

The main strength of the study is the state-of-the-art methods used concurrently to assess retinopathy, nephropathy and particularly neuropathy. We confirm CCM identifies small nerve fibre regeneration following bariatric surgery, demonstrating the utility of CCM as a surrogate end point for the assessment of nerve fibre repair.

The limitations of this study include the small sample size and lack of a matched control group of patients with type 2 diabetes who have not undergone bariatric surgery. Also, due to the small number of patients who underwent sleeve gastrectomy as opposed to gastric bypass we cannot adequately test for procedure specific effects. NCS were only performed in 9 participants, primarily due to technical difficulties in performing NCS because of excess subcutaneous adipose tissue in these patients.

In conclusion, we show for the first time that bariatric surgery can potentially lead to an early reversal of diabetic neuropathy, particularly small fibre pathology. We also report a beneficial effect on glomerular hyperfiltration, but no impact on albuminuria or retinopathy. These improvements may be driven by an improvement in weight, SBP and triglycerides, which warrant further study.

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Declaration of Interests

H.S. has received grants and personal fees from Akcea, grants and personal fees from Amgen, grants from Pfizer, grants and personal fees from MSD, personal fees from Sanofi, personal fees from Synageva, personal fees from Takeda, which are all outside of the submitted work. R.M has received grants and personal fees from Pfizer and Novo Nordisk. None of the other authors have any relevant conflicts of interest to declare.

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Chapter 8: Male sexual dysfunction in obesity: The role of sex hormones and small fibre neuropathy

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RESEARCH ARTICLE

Male sexual dysfunction in obesity: The role of sex hormones and small fibre neuropathy

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Abstract

Context

Multiple factors contribute to sexual dysfunction in men with obesity. Sex hormone levels are commonly abnormal in men with obesity and this abnormality is often the focus of management in clinical practice. The role of small fibre neuropathy in obesity-related sexual dysfunction is not well established.

Objective

We aimed to investigate the relationship between sexual function, sex hormone levels and small nerve fibre morphology in men with severe obesity.

Materials and methods

A prospective study of 29 men with severe obesity was undertaken. Sexual function was assessed using the European Male Ageing Study Sexual Function Questionnaire. Small nerve fibre morphology was quantified using corneal confocal microscopy. Sex hormone levels were measured by mass spectrophotometry.

Results

Erectile dysfunction was present in 72% of the cohort with a higher prevalence of diabetes among the symptomatic group (88% vs 38%, $p = 0.006$). Corneal nerve fibre length (CNFL) and corneal nerve fibre density (CNFD) were both significantly lower in participants with erectile dysfunction compared to those without ($p = 0.039$ and $p = 0.048$ respectively). The erectile function score correlated with CNFL ($r = -0.418$, $p = 0.034$) and CNFD ($r = -0.411$, $p = 0.037$). Total testosterone and calculated free testosterone levels did not differ significantly between men with or without erectile dysfunction (median 8.8 nmol/L vs 9.0 nmol/L, $p = 0.914$; and median 176 pmol/L vs 179 pmol/L, $p = 0.351$ respectively), infrequent sexual

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thoughts (median 8.1 nmol/L vs 9.2 nmol/L, p = 0.650; and median 184 pmol/L, vs 176 pmol/L, p = 0.619 respectively) and decreased morning erections (median 9.0 nmol/L vs 8.8 nmol/L, p = 0.655; and median 170 pmol/L vs 193 pmol/L, p = 0.278 respectively).

Conclusion

Sexual dysfunction is highly prevalent in men with severe obesity. We found an association between small fibre neuropathy with erectile dysfunction with presence of diabetes a likely a significant contributing factor. We found no associations between testosterone levels with sexual symptoms (including frequency of sexual thoughts). The influence of small nerve fibre neuropathy on response to therapeutic interventions and whether interventions that improve small fibre neuropathy can improve erectile function in this population merits further study.

Introduction

Sexual dysfunction is common in men with obesity [1]. Normal sexual functioning is dependent on complex vascular, neural, hormonal and psychological factors, all of which are potentially affected by obesity [2, 3]. Low testosterone level, in particular, is thought to have a bidirectional relationship with obesity [4, 5] and is common in men with obesity [6] and is very common in men with severe obesity [7, 8]. Current guidelines recommend testosterone replacement in men with symptomatic androgen deficiency to improve general well-being, bone mineral density and sexual function [9]. Whilst testosterone therapy may improve sexual symptoms in hypogonadal men [9], evidence that it benefits sexual function in men with obesity and low levels of testosterone is inconsistent [10–13].

Erectile dysfunction is associated with diminished small and large nerve fibre sensory thresholds [14]. Peripheral neuropathy, especially small fibre neuropathy, occurs more commonly in people with obesity than in those without [15, 16]. Corneal confocal microscopy is a rapid, validated and non-invasive technique that assesses small nerve fibre integrity [17, 18] with comparable diagnostic efficiency to intraepidermal nerve fibre density in diabetic peripheral neuropathy [19]. We have recently shown that small fiber neuropathy, quantified using corneal confocal microscopy, is associated with erectile dysfunction in men with type 1 diabetes [20].

We aimed to assess, for the first time, whether relationships exist between sexual symptoms with small fibre neuropathy and/or with sex hormone levels in men with severe obesity.

Materials and methods

Participants

Twenty-nine male patients with severe obesity were recruited from the weight management clinic at Salford Royal National Health Service Foundation Trust (Salford, United Kingdom). Severe obesity is defined as BMI above 40 which is equivalent to obesity class III using the BMI classification set out by the World Health Organisation (WHO) [21]. Patients known to have cardiovascular disease, disease of the pituitary, testes or adrenal glands, or taking therapy for erectile dysfunction or known to affect androgen levels or cause erectile dysfunction and luteinising hormone >9.4 U/L (primary hypogonadism), were excluded [22]. Further exclusion criteria included: other causes of peripheral neuropathy apart from diabetes; cancer, radiotherapy or chemotherapy; previous eye surgery and corneal disease. Presence of comorbidities

such as hypertension and type 2 diabetes were determined on medical history. A HbA1c measurement at baseline was used in addition to identify patients with undiagnosed type 2 diabetes (HbA1c $\geq$ 48 mmol/mol) and pre-diabetes (HbA1c 42–47 mmol/mol) [23]. This study has approval from the Greater Manchester Central Research and Ethics Committee. Written informed consent was obtained from all individuals prior to participation.

Sexual function assessment

Sexual function was assessed using the European Male Ageing Study Sexual Function Questionnaire [24]. Three sexual symptoms were used for assessment of sexual function: erectile function, frequency of sexual thoughts, and frequency of morning erections. Participants were divided into symptomatic and asymptomatic groups using previously established cut-offs based on validated scores for each individual question relating to the three sexual symptoms [25, 26].

Neuropathy assessment

Symptoms of peripheral neuropathy was assessed using the neuropathy symptom profile (NSP). Cold (CT) and warm (WT) perception thresholds were assessed on the dorsolateral aspect of the left foot (S1) using the TSA-II NeuroSensory Analyser (Medoc, Ramat-Yishai, Israel). Electrodiagnostic studies were undertaken using a Dantec Keypoint system (Dantec Dynamics, Bristol, UK). Vibration perception threshold (VPT) was established using a Horwell Neurothesiometer (Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Deep breathing heart rate variability (DB-HRV) was established using an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies, Philadelphia, PA, USA).

Corneal confocal microscopy

Corneal nerve morphology was assessed using corneal confocal microscopy [17, 27]. Corneal confocal microscopy (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) was performed using our established protocol [27]. Six non-overlapping images from the centre of the cornea were selected per patient (three per eye) [28]. Automated image analysis was performed using ACCMetrics software (The University of Manchester, United Kingdom). Corneal nerve fibre length (total length of nerves in mm per mm^2), corneal nerve fibre density (CNFD) (number of major nerves per mm^2) and corneal nerve branch density (CNBD) (number of nerve branches per mm^2) were quantified [29].

Laboratory measurements

Venous blood samples were obtained between the hours of 0800 and 1100 after an overnight fast of at least 12 hours. Apart from glycosylated haemoglobin (HbA1c) which was measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust on the day of collection, all other laboratory measurements were performed at the end of the study. Serum or plasma, isolated within 2 hours of collection, was stored at 4˚C or -20˚C until analysed. Each serum aliquot was stored for a maximum of two years and underwent one freeze-thaw cycle only. Serum total testosterone, dihydrotestosterone, dehydroepiandrosterone sulphate and androstenendione levels were determined using liquid chromatography–tandem mass spectrometry in a validated clinical laboratory [30, 31]. Sex hormone-binding globulin, luteinising hormone and follicle-stimulating hormone levels were measured electrochemiluminescence immunoassay (Roche Diagnostics) using Roche® automated analysers (E170 platform). Serum free testosterone levels were calculated using the mass action equation described by Vermeulen [32] and participant-specific total testosterone,

sex hormone-binding globulin and albumin levels. A participant was considered to have a low testosterone level if either; his total testosterone level was less than 8 nmol/L; or his total testosterone level was between 8 and 11 nmol/L *and* his calculated free testosterone level was less than 220 pmol/L [25].

Statistical analyses

All statistical analyses were performed using SPSS for Windows (Version 23.0, IBM SPSS Statistics, Armonk, NY). Continuous variables were compared between groups using the independent samples t-test or, in the case of non-parametrically distributed data, the Mann-Whitney U test. Normality of data distribution was assessed for all continuous variables using the Shapiro-Wilk test. The chi-squared test was used for analysis of categorical data. Correlations between variables were assessed using Spearman's analyses. Results are expressed as mean with standard deviation (SD) for parametric data and as median with interquartile range (IQR) for non-parametric data. No attempt was made to adjust for missing data. The level of statistical significance was set at less than 0.05 for all analyses.

Results

Sixteen (55%) of the participants had erectile dysfunction of whom 34% had severe erectile dysfunction. Infrequent sexual thoughts and decreased morning erections were reported in 13 (45%) and 23 (79%) of the participants respectively. The median overall satisfaction score corresponded with moderate dissatisfaction.

The median total testosterone was 9.0 nmol/L, 95% confidence interval ranged between 7.4 nmol/L and 11.1 nmol/L. The total testosterone level was less than 8 nmol/L in 12 (41%) participants, between 8 and 11 nmol/L in ten (34%) participants, of whom 4 (14%) had a calculated free testosterone level below 220 pmol/L.

Erectile dysfunction

When participants were divided into symptomatic and asymptomatic groups based on their erectile function scores, age was higher and body mass index was lower in the symptomatic group (Table 1). The prevalence of type 2 diabetes mellitus and hypertension were higher in those with erectile dysfunction. The HbA1c, however, did not differ significantly between the groups. The prevalence of dysglycaemia (pre-diabetes and type 2 diabetes mellitus) also did not differ between symptomatic and asymptomatic groups.

Both CNFL and CNFD were lower in participants with erectile dysfunction compared to those without (Table 2 and Fig 1). Erectile function score correlated with both CNFL (Spearman's r = -0.418, p = 0.034) and CNFD (Spearman's r = -0.411, p = 0.037).

The level of total testosterone, free testosterone, sex hormone-binding globulin, dihydrotestosterone, dehydroepiandrosterone sulphate and androstenendione did not differ between participants with and without erectile dysfunction (Table 1 and Fig 1). There was no difference in the prevalence of low testosterone between these two groups. Total and free testosterone did not correlate with erectile function (Spearman's r = -0.083, p = 0.667 and Spearman's r = -0.255, p = 0.181 respectively).

There were no difference in lipid profile between the two groups.

Infrequent sexual thoughts and decreased morning erections

Clinical, corneal nerve or hormone parameters did not differ between those with and without infrequent sexual thoughts (S2 Table) or in those with and without decreased morning

Table 1. Comparison of demographics, clinical characteristics, and sex hormone levels between asymptomatic and symptomatic patients divided based on erectile function.

	Asymptomatic based on erectile function (n = 13)	Symptomatic based on erectile function (n = 16)	p-value
Clinical characteristics			
Age, years	44.4 ±8.4	52.1 ±10.9	**0.045**
BMI, kg/mm^2	54.8 ±12.4	46.9 ±7.3	**0.041**
Type 2 diabetes, n (%)	5 (38%)	14 (88%)	**0.006**
Duration of diabetes, years	5±3	6±5	0.787
Pre-diabetes and type 2 diabetes, n (%)	10 (77%)	14 (88%)	0.453
Hypertension, n (%)	4 (30%)	11 (68%)	**0.042**
Antihypertensives, n	0 (0–1)	1 (0–2)	0.092
Biochemistry			
HbA1c, mmol/mol	52±13	55±15	0.591
Total cholesterol, mmol/l	3.8±1.2	4.0±1.0	0.796
Triglyceride, mmol/l	1.0±0.5	1.3±0.5	0.206
HDL-C, mmol/l	1.01±0.38	0.95±0.22	0.702
LDL-C, mmol/l	2.5±1.0	2.4±0.8	0.808
Sex hormones			
Low testosterone, n (%)	7 (54)	10 (63)	0.638
Total testosterone, nmol/L	9.0 (6.4–12.3)	8.8 (6.4–11.0)	0.914
Free testosterone, pmol/L	179 (132–311)	176 (120–216)	0.351
Sex hormone-binding globulin, nmol/L	29.2 (21.7–35.5)	32.1 (21.7–38.3)	0.559
Luteinising hormone, mIU/Ml	2.3±1.2	3.5±1.9	0.059
Follicle-stimulating hormone, mIU/L	4.2 (2.9–4.7)	3.5 (2.4–4.9)	0.779
Dihydrotestosterone, nmol/L	0.60 (0.34–0.98)	0.62 (0.50–0.99)	0.914
Dehydroepiandrosterone sulphate, nmol/L	2.2 (1.1–3.4)	1.3 (0.9–3.6)	0.537
Androstenedione, nmol/L	2.3 (1.6–3.0)	1.6 (1.3–2.4)	0.170

Notes: Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Independent t-test was performed for normally-distributed variables, Mann-Whitney U test for non-parametric variables, and chi-squared test for categorical variables when comparing asymptomatic and symptomatic groups. $p<0.05$ is considered statistically significant.

Abbreviations: BMI, body mass index; HbA1c, glycosylated haemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

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erections (S3 Table). Total and free testosterone did not correlate with frequency of sexual thoughts (Spearman's r = 0.236, p = 0.218 and Spearman's r = 0.179, p = 0.352 respectively) or with frequency of morning erections (Spearman's r = 0.079, p = 0.682 and Spearman's r = 0.186, p = 0.333 respectively). The HbA1c and lipid profile did not differ between patients with and without infrequent sexual thoughts and patients with and without decreased morning erections (S2 and S3 Tables).

Low testosterone

There was no difference in sexual symptom frequency between men with and without a low testosterone level (S4 Table).

Discussion

This is the first study to assess sexual function, small fibre neuropathy, sex hormone levels and their relationships simultaneously in men with severe obesity. Male sexual dysfunction was

Table 2. Comparison of measures of neuropathy between asymptomatic and symptomatic patients divided based on erectile function.

	Asymptomatic based on erectile function (n = 13)	Symptomatic based on erectile function (n = 16)	p-value
NSP, /38	0 (0–5)	4 (0–15)	0.131
VPT, V	13.3±5.6	16.3±7.2	0.296
CT, ˚C	22.6±5.9	20.6±8.2	0.528
WT, ˚C	41.0±2.3	41.9±3.3	0.437
CNFL, mm/mm^2	20.29±3.21	16.74±4.45	**0.039**
CNFD, no./mm^2	30.21 (27.34–33.59)	27.60 (22.50–29.17)	**0.048**
CNBD, no./mm^2	60.75±33.53	45.70±24.86	0.201
DB-HRV, beats/min	35 (24–44)	14 (12–23)	**0.016**

Notes: Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Independent t-test was performed for normally-distributed variables, Mann-Whitney U test for non-parametric variables, and chi-squared test for categorical variables when comparing asymptomatic and symptomatic groups. $p<0.05$ is considered statistically significant.

Abbreviations: CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; CT, cold perception threshold; DB-HRV, deep breathing heart rate variability; NSP, neuropathy symptom profile; VPT, vibration perception threshold; WT, warm perception threshold.

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highly prevalent in our study population with symptomatic erectile dysfunction and reduction in frequency of sexual thoughts being present in 55% and 45% of patients, respectively. The vast majority of patients had overall sexual function scores below the mean value found in the European Male Ageing Study, despite being ten years younger (mean age of 49 compared to

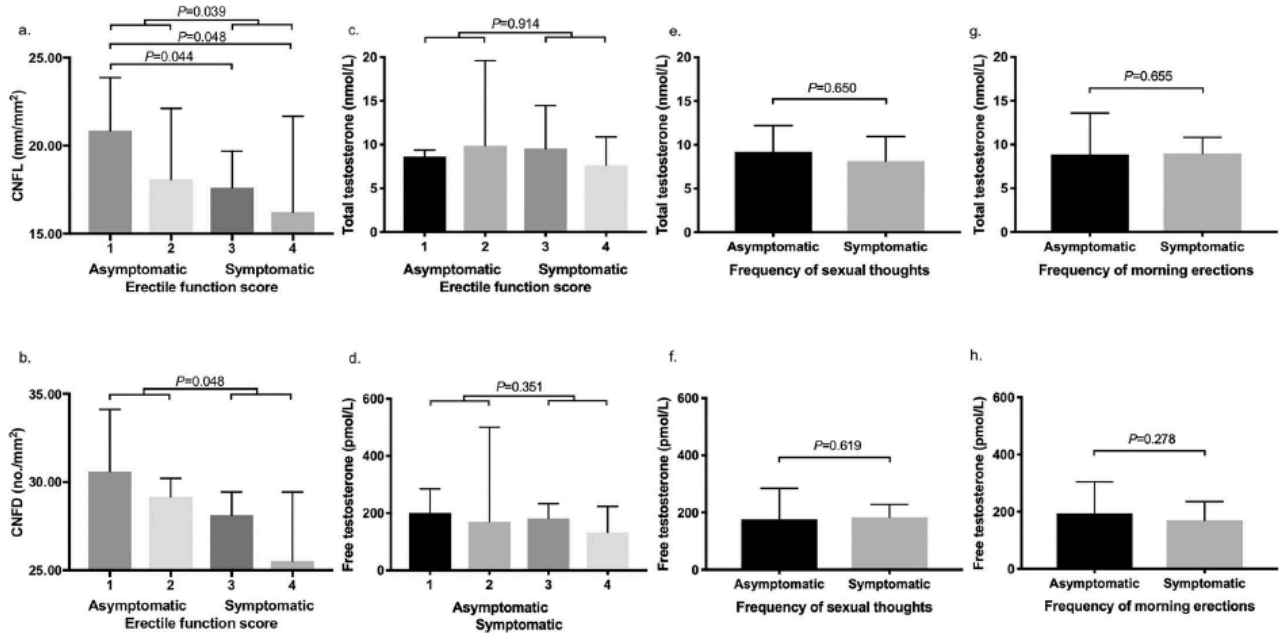

Fig 1. Comparison of corneal nerve parameters in asymptomatic and symptomatic patients based on erectile function score (a & b) and comparison of total and free testosterone levels between asymptomatic and symptomatic patients based on erectile function (c & d), frequency of sexual thoughts (e & f), and frequency of morning erections (g & h). CNFL and CNFD are both significantly lower in symptomatic compared to asymptomatic patients with erectile dysfunction (a & b). Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Erectile function questionnaire response categories: 1: always able to keep erection good enough for sexual intercourse, 2: usually able, 3: sometimes able, 4: never able. Abbreviations: CNFD, corneal nerve fibre density; CNFL, corneal nerve fibre length. $p<0.05$ is considered statistically significant.

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59 years) [24]. The level of distress related to sexual functioning was also higher compared to that observed in the European Male Ageing Study cohort.

We have demonstrated, for the first-time, that reduction in small nerve fibre indices occur in patients with severe obesity and erectile dysfunction. In particular, corneal nerve fibre density and length were significantly lower in patients with symptomatic erectile dysfunction and they correlated with erectile function. There was, however, no difference between corneal nerve branch density and the severity of erectile dysfunction. We hypothesise that this could be a result of constant changes in this parameter secondary to nerve regeneration. There was also evidence of autonomic dysfunction in patients with erectile dysfunction. Older age, obesity and a higher prevalence of diabetes and hypertension in the cohort with erectile dysfunction are likely to be the major contributors of corneal nerve loss in accord with our previous studies [33, 34] and our recent study in type 1 diabetes.

Although somatic and autonomic neuropathy are associated with erectile dysfunction [35], patients continue to have assessment of sex hormones and cardiovascular risk factors, but not neuropathy. Our study emphasizes the importance of assessing small fibre neuropathy as it plays an important role in the neurovascular regulation of erectile function [36, 37]. Although phosphodiesterase type 5 inhibitors (PDE5i) are used in the management of erectile dysfunction, they are less effective in people with nerve damage [38, 39]. Based on our study findings, we therefore suggest that assessment of small fibre neuropathy prior to initiation of PDE5i may help identify patients who are more likely to respond to treatment and corneal confocal microscopy provides a rapid, objective and clinically feasible method to assess small fibre damage and repair [17–19]. Larger studies will be required to prove this further.

In keeping with previously published studies, we found a high prevalence of low testosterone levels in this cohort of men with severe obesity [8]. However, testosterone levels were not associated with sexual symptoms and sexual symptom frequency did not differ between those with low and normal testosterone levels. This perhaps suggests that low testosterone might not be a major determinant of presence of sexual symptoms in severe obesity.

The limitations of this study are the cross-sectional design which limits the inference of cause and effect between small fibre neuropathy and erectile dysfunction. A larger sample size would have allowed adjustment of confounding factors for small fibre damage in relation to erectile dysfunction. A single sample to determine sex hormone levels may also be seen as a limitation, however, several studies have confirmed that testosterone levels do not fluctuate significantly when measured serially in a timed sample over several months [40–42].

Conclusion

We conclude that erectile dysfunction is common among men with severe obesity and is associated with small fibre neuropathy, likely to be further driven by older age, diabetes and cardiovascular risk factors. Corneal confocal microscopy is a rapid, non-invasive technique to quantify of small nerve fibre degeneration and regeneration. Prospective studies are required to assess the impact of small fibre neuropathy on the effectiveness of therapies for erectile dysfunction and whether interventions that improve small fibre neuropathy can improve erectile dysfunction in men with severe obesity.

Supporting information

S1 Table. Questions regarding sexual symptoms within the EMAS sexual function questionnaire and definitions of asymptomatic and symptomatic response categories. The

definitions of asymptomatic and symptomatic response categories are based on validated published criteria [25, 26].
(DOCX)

S2 Table. Comparison of sex hormone levels between asymptomatic and symptomatic patients divided based on frequency of sexual thoughts. Notes: Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Independent t-test was performed for normally-distributed variables, Mann-Whitney U test for non-parametric variables, and chi-squared test for categorical variables. $p<0.05$ is considered statistically significant. **Abbreviations:** CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length.
(DOCX)

S3 Table. Comparison of sex hormone levels between symptomatic and asymptomatic patients divided based on frequency of morning erections. Notes: Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Independent t-test was performed for normally-distributed variables, Mann-Whitney U test for non-parametric variables, and chi-squared test for categorical variables. $p<0.05$ is considered statistically significant. **Abbreviations:** CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length.
(DOCX)

S4 Table. Comparison of sexual symptoms between groups with low testosterone and normal testosterone. Data are presented as median and interquartile range for non-parametric variables. Mann-Whitney U test was performed for non-parametric variables. Questionnaire response categories: erectile function: 1: always able to keep erection good enough for sexual intercourse, 2: usually able, 3: sometimes able, 4: never able; frequency of sexual thoughts and morning erection frequency: 1: none or once in the past month, 2: 2–3 times/month and 1 time/week, 3: 2–6 times/week, 4: $\geq$1/day; overall satisfaction: 0: very dissatisfied, 1: moderately dissatisfied, 2: equally satisfied and dissatisfied, 3: moderately satisfied, 4: very satisfied. Overall sexual function score ranges from 0 to 33 with higher scores corresponding with higher level of sexual functioning. Sexual functioning-related distress ranges from 0 to 20, with higher scores corresponding with higher level of distress. $p<0.05$ is considered statistically significant.
(DOCX)

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Supplemental Material

S1 Table. Questions regarding sexual symptoms within the EMAS sexual function questionnaire and definitions of asymptomatic and symptomatic response categories.

	Asymptomatic	**Symptomatic**
Were you able to get and keep an erection sufficient for sexual intercourse?	Usually or always	Never or sometimes
How often did you think about sex?	Once a week or more	2–3 times in the past month
How frequently did you awaken with a full erection in the past month?	2–3 times in the past month	≤1 time in the past month

The definitions of asymptomatic and symptomatic response categories are based on validated published criteria.

S2 Table. Comparison of sex hormone levels between asymptomatic and symptomatic patients divided based on **frequency of sexual thoughts**.

	Asymptomatic based on frequency of sexual thoughts (n=16)	**Symptomatic based on frequency of sexual thoughts (n=13)**	***P*-value**
Clinical characteristics			
Age, years	45.9±10.2	52.0±10.0	0.122
Body mass index, kg/mm^2	51.4±11.1	49.3±9.7	0.613
Type 2 diabetes, n (%)	9 (56%)	14 (76%)	0.244
Duration of diabetes, years	5±4	6±4	0.716
Hypertension, n (%)	8 (50%)	7 (54%)	0.837
Antihypertensives, n	0 (0–1)	1 (0–2)	0.983
Biochemistry			
HbA1c, mmol/mol	49 (42–61)	51 (42–60)	0.982
Total cholesterol, mmol/l	3.5±0.8	4.1±1.0	0.173
Triglyceride, mmol/l	1.1±0.6	1.3±0.4	0.256
HDL-C, mmol/l	1.03±0.38	0.94±0.20	0.499
LDL-C, mmol/l	2.0±0.5	2.5±0.9	0.195
Sex hormones			
Low testosterone, n (%)	10 (63%)	7 (54%)	0.716
Total testosterone, nmol/L	9.2 (6.9–12.2)	8.1 (5.5–11.0)	0.650
Free testosterone, pmol/L	176 (135–285)	184 (107–229)	0.619
Sex hormone-binding globulin, nmol/L	32.1 (24.1–38.7)	28.0 (20.6–33.5)	0.374

Luteinising hormone, mIU/mL	3.0±1.9	3.1±1.7	0.930
Follicle-stimulating hormone, mIU/L	3.5±1.8	3.7±1.3	0.720
Dihydrotestosterone, nmol/L	0.61 (0.39–1.03)	0.60 (0.50–0.85)	0.650
Dehydroepiandrosterone sulphate, nmol/L	2.2 (1.1–3.4)	1.2 (0.8–3.9)	0.537
Androstenedione, nmol/L	2.4 (1.6–3.1)	1.6 (1.3–2.2)	0.050
Corneal nerve parameters			
CNFL, mm/mm^2	18.41±3.93	17.81±4.82	0.733
CNFD, no./mm^2	28.12 (26.04–30.21)	28.12 (22.92–31.77)	0.880
CNBD, no./mm^2	49.85±33.00	53.12±25.23	0.779

Notes: Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Independent t-test was performed for normally-distributed variables, Mann-Whitney U test for non-parametric variables, and chi-squared test for categorical variables. $p<0.05$ is considered statistically significant.

Abbreviations: CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length.

S3 Table. Comparison of sex hormone levels between symptomatic and asymptomatic patients divided based on **frequency of morning erections**.

	Asymptomatic based on frequency of morning erections (n=6)	**Symptomatic based on frequency of morning erections (n=23)**	***P*-value**
Clinical characteristics			
Age, years	52.0±9.2	47.8±10.8	0.388
Body mass index, kg/mm^2	47.6±6.3	51.2±11.3	0.460
Type 2 diabetes, n (%)	5 (83%)	14 (61%)	0.303
Duration of diabetes, years	5±4	6±4	0.663
Hypertension, n (%)	4 (67%)	11 (48%)	0.411
Biochemistry			
HbA1c, mmol/mol	67±15	49±11	**0.033**
Total cholesterol, mmol/l	3.7±0.4	4.0±1.1	0.662
Triglyceride, mmol/l	1.5±0.6	1.2±0.5	0.357
HDL-C, mmol/l	0.98±0.41	0.97±0.23	0.918
LDL-C, mmol/l	2.2±0.2	2.5±0.9	0.598
Sex hormones			
Low testosterone, n (%)	3 (50%)	14 (61%)	0.630
Total testosterone, nmol/L	8.8 (6.9–13.6)	9.0 (6.2–10.8)	0.655
Free testosterone, pmol/L	193 (166–305)	170 (116–235)	0.278
Sex hormone-binding globulin, nmol/L	24.7 (15.6–39.6)	31.8 (22.0–37.6)	0.302
Luteinising hormone, mIU/mL	2.8±2.1	3.1±1.7	0.747

Follicle-stimulating hormone, mIU/L	3.2±2.1	3.7±1.4	0.501
Dihydrotestosterone, nmol/L	0.61 (0.39–1.03)	0.60 (0.50–0.85)	0.813
Dehydroepiandrosterone sulphate, nmol/L	2.2 (1.1–3.4)	1.2 (0.8–3.9)	0.059
Androstenedione, nmol/L	2.4 (1.6–3.1)	1.6 (1.3–2.2)	0.854
CNFL, mm/mm^2	19.27±3.58	17.76±4.54	0.465
CNFD, $no./mm^2$	28.97±3.51	26.46±6.49	0.376
CNBD, $no./mm^2$	47.95±23.51	52.55±30.71	0.739

Notes: Data are presented as mean and standard deviation for normally-distributed variables and median and interquartile range for non-parametric variables. Independent t-test was performed for normally-distributed variables, Mann-Whitney U test for non-parametric variables, and chi-squared test for categorical variables. $p<0.05$ is considered statistically significant.

Abbreviations: CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length.

S4 Table. Comparison of sexual symptoms between groups with low testosterone and normal testosterone.

	Normal testosterone (n=12)	**Low testosterone (n=17)**	***P*-value**
Erectile function	3 (1–4)	3 (2–4)	0.711
Frequency of sexual thoughts	3 (1–5)	3 (0–5)	0.811
Frequency of morning erections	0 (0–2)	0 (0–1)	0.845
Overall sexual function	10 (4–15)	10 (0–16)	0.777
Sexual functioning-related distress	3 (2–11)	6 (2–10)	0.499
Overall satisfaction	1 (0–2)	0 (0–2)	0.556

Data are presented as median and interquartile range for non-parametric variables. Mann-Whitney U test was performed for non-parametric variables.

Questionnaire response categories: erectile function: 1: always able to keep erection good enough for sexual intercourse, 2: usually able, 3: sometimes able, 4: never able; frequency of sexual thoughts and morning erection frequency: 1: none or once in the past month, 2: 2–3 times/month and 1 time/week, 3: 2–6 times/week, 4: ≥1/day; overall satisfaction: 0: very dissatisfied, 1: moderately dissatisfied, 2: equally satisfied and dissatisfied, 3: moderately satisfied, 4: very satisfied.

Overall sexual function score ranges from 0 to 33 with higher scores corresponding with higher level of sexual functioning.

Sexual functioning-related distress ranges from 0 to 20, with higher scores corresponding with higher level of distress.

$p<0.05$ is considered statistically significant.

Chapter 9: Vitamin D status after gastric bypass or sleeve gastrectomy over 4 years of follow-up

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Vitamin D status after gastric bypass or sleeve gastrectomy over 4 years of follow-up

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Abstract

Background: Bariatric surgery for severe obesity can lead to micronutrient/vitamin deficiencies.
Aims: To study baseline and post-surgical prevalence of vitamin D deficiency in patients undergoing bariatric surgery.
Participants and Setting: Patients undergoing bariatric surgery in a university teaching hospital in North-West England.
Methods: We performed an observational cohort analysis of longitudinal data on vitamin D and related parameters in patients who underwent bariatric surgery. Patients were routinely recommended daily combined calcium and vitamin D supplementation post-surgery.
Results: We studied 460 patients who had completed at least 12 months postoperative follow-up; mean (standard deviation) age was 48.0 (10.5) years, weight 144.7 (27.3) kg and body mass index 50.0 (7.6) kg/m^2; 292 (63.5%) underwent gastric bypass and 168 (36.5%) sleeve gastrectomy. Vitamin D level was 33.1 (23.9) nmol/L at baseline, rising to 57.1 (23.1) nmol/L at 12 months post-surgery. Whereas 43.2% had vitamin D deficiency and 34.7% insufficiency preoperatively, 8.9% and 26.7% had deficiency and insufficiency, respectively, at 12 months with similar trends up to 4 years of follow-up. There were no significant differences between procedures or sexes in vitamin D levels or sufficiency rates.
Conclusion: Vitamin D deficiency and insufficiency were prevalent pre-surgery and reduced significantly with routine supplementation post-surgery.

Key words: Bariatric surgery; Vitamin D Deficiency; Parathyroid Hormone; Hyperparathyroidism; Calcium;

Introduction

Obesity is a major public health concern of our times. Whilst lifestyle, dietary and behavioural changes and weight loss pharmacotherapy remain key interventions, bariatric surgery is the most effective treatment for severe obesity. It has been demonstrated that it leads to clinically significant weight loss and is cost-effective (1–4). Post-bariatric surgery, patients typically achieve substantial weight loss and significant improvement or resolution of weight-related comorbidities including Type 2 diabetes mellitus (T2DM), hypertension and hyperlipidaemia. A recent meta-analysis of observational data reported that patients who underwent bariatric surgery had 50% lower mortality than controls over follow-up of 2 to 15 years (5). Therefore, bariatric surgery is widely recommended as a treatment for severe obesity (6). Bariatric procedures are associated with various micronutrient deficiencies including vitamin D deficiency (7–11). Vitamin D is important for calcium homeostasis and bone metabolism. There are concerns that bone health may be adversely affected by bariatric surgery, and that vitamin D deficiency may play a part (12–18). One meta-analysis of 51 studies found mean vitamin D levels ≤ 20 ng/mL (50 nmol/L) in a third of studies (10); mean vitamin D levels remained ≤ 30 ng/mL (75 nmol/L) following bariatric surgery despite various vitamin D replacement regimens, with few exceptions. Another meta-analysis of 12 studies found that a daily vitamin D supplement of > 800 IU (but not < 800 IU) significantly reduced the prevalence of post-operative vitamin D depletion at 1 year (19). Our centre recommends that patients take ≥ 800 IU vitamin D along with 1000–1200 mg calcium daily consistent with the British Obesity & Metabolic Surgery Society guideline [8]. Patients are encouraged to attend follow-up appointments for life-long monitoring, and supplement doses are adjusted according to requirements. We now report a longitudinal cohort analysis of vitamin D and associated outcomes of bariatric surgery over 4 years of follow-up.

Methods

We performed an observational cohort analysis of prospectively recorded longitudinal data on vitamin D and related parameters in patients who underwent bariatric surgery.

Patients and setting

We studied patients who had undergone primary bariatric surgery over a four-year follow-up period at a university teaching hospital in the North-West of England, UK. Permission was obtained from the Clinical Audit department of our institution. Patient selection for primary bariatric surgery was in accordance with the National Institute for Health and Clinical Excellence (NICE) clinical guideline and the National Health Service (NHS) clinical commissioning policy for complex and severe obesity (6,20). Patients who had undergone gastric bypass or sleeve gastrectomy were included; other primary bariatric surgeries, such as gastric banding, and revisional procedures were excluded. Patients who were undergoing another surgical procedure at the same time as gastric bypass or sleeve gastrectomy (e.g. laparoscopic cholecystectomy or hernia repair along with primary bariatric surgery) were not excluded.

A total of 460 patients who underwent primary bariatric surgery between March 2012 – March 2016 were included, and data were extracted from electronic patient records in April 2017 such that all patients had completed 12 months postoperatively; the number of patients who had completed 24 months, 36 months and 48 months was 362, 253 and 149, respectively. Data collected included patient demographics, preoperative baseline data and postoperative follow-up data, including weight, height, body mass index (BMI; kg/m^2) and percent total weight loss (%TWL); and blood tests including total vitamin D (25-hydroxyvitamin D, 25(OH)D), phosphate, alkaline phosphatase (ALP), parathyroid hormone (PTH), albumin and total calcium. The laboratory recommended reference ranges for the time period of study were used (21). The calcium level was adjusted for serum albumin < 40 g/L by applying the formula, *adjusted calcium (mmol/L) = total calcium (mmol/L) + 0.02*[40 –*

albumin (g/L)] (22). Vitamin D levels were categorised using the thresholds recommended by the National Osteoporosis Society as deficient (25(OH)D < 25 nmol/L), insufficient (25–50 nmol/L) or sufficient (> 50 nmol/L) (23). Glycated haemoglobin (HbA1c) was also recorded as a metabolic disease measure.

Statistical analysis

We performed descriptive statistics of demographic characteristics with parametric tests (or non-parametric tests for non-normative data), with measures of dispersion as appropriate. Comparative analyses were performed with independent or paired samples t-tests as appropriate. The Fisher exact test was used to analyse contingency tables of categorical variables. Linear associations were analysed with the Pearson correlation formula. A two-sided $P < 0.05$ was considered statistically significant. To cope with missing values because of the retrospective, observational nature of the study frequencies were reported as valid percentages and significant results were confirmed by re-analysing data after multiple imputation by the fully conditional specification method. Results were reported based on original, unimputed data. Data were analysed using IBM SPSS Statistics 23.0 (IBM Corp, Armonk, NY) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

Results

We studied longitudinal vitamin D status in patients who underwent primary bariatric surgery.

Patient demographics

The 460 patients comprised 299 women (65%) and 161 men (35%). The mean (standard deviation; SD) age at time of surgery was 48.0 (10.5) years. Baseline weight was 144.7 (27.3) kg and BMI 50.0 (7.6) kg/m². Gastric bypass was the most frequent operation (292, 63.5%), followed by sleeve gastrectomy (168, 36.5%).

Weight loss (and metabolic) outcomes

Following bariatric surgery there was significant weight loss, with mean (SD) reduction in BMI of 14.7 (4.8) kg/m^2 ($P < 0.001$) at 12 months (**Table 1**). The weight loss was sustained over 4 years of follow-up, with a non-significant rise in BMI from 35.1 (6.6) kg/m^2 at 12 months to 37.2 (7.2) kg/m^2 at 48 months. The weight loss equated to %TWL of 31.4 (8.1) % at 12 months (**Figure 1 A**). The %TWL for gastric bypass vs. sleeve gastrectomy was 32.6% vs. 29.4% ($P < 0.001$) at 12 months, 35.5% vs. 29.1% ($P < 0.001$) at 24 months, 33.5% vs. 27.0% ($P < 0.006$) at 36 months and 30.8% vs. 25.8% (non-significant; ns) at 48 months. There were no consistent significant differences in weight loss between men and women or people with or without diabetes.

Table 1: Outcomes after bariatric surgery

Measurements (recommended range)	0 months	12 months	24 months	36 months	48 months
Body mass index (18.5 – 24.9 kg/m^2)	50.0 (7.6) (444/460)‡	35.1 (6.6)*** (363/460)‡	34.3 (6.8)*** (200/362)‡	36.2 (7.0)*** (99/253)‡	37.2 (7.2) (52/149)‡
HbA1c (≤ 41 mmol/mol)†	61.6 (20.3) (180/189)‡	39.6 (7.9)*** (143/189)‡	39.5 (11.0) (75/146)‡	42.5 (13.6) (48/93)‡	42.5 (10.6) (22/52)‡
Vitamin D (50 – 125 nmol/L)	33.1 (23.9) (303/460)‡	57.1 (23.1)*** (326/460)‡	57.5 (24.3) (176/362)‡	51.2 (23.0)*** (98/253)‡	54.0 (22.1) (59/149)‡
Parathyroid hormone (1.5 – 7.6 pmol/L)	5.8 (3.3) (315/460)‡	5.9 (2.9) (336/460)‡	6.1 (3.3) (183/362)‡	6.2 (3.0) (93/253)‡	6.2 (3.0) (48/149)‡
Adjusted calcium (2.20 – 2.60 mmol/L)	2.33 (0.10) (423/460)‡	2.30 (0.11)*** (359/460)‡	2.28 (0.09)*** (200/362)‡	2.26 (0.09)** (105/253)‡	2.27 (0.09) (59/149)‡
Albumin (35 – 50 g/L)	43.5 (3.0) (456/460)‡	41.8 (3.4) (364/460)‡	42.1 (3.4) (201/362)‡	42.2 (2.5) (107/253)‡	41.7 (3.0) (59/149)‡
Phosphate (0.8 – 1.5 mmol/L)	1.05 (0.18) (416/460)‡	1.13 (0.17) (353/460)‡	1.09 (0.16) (198/362)‡	1.07 (0.14) (105/253)‡	1.06 (0.19) (58/149)‡
Alkaline phosphatase (30 – 130 U/L)	84.9 (27.4) (454/460)‡	86.2 (29.3) (362/460)‡	81.9 (25.9) (200/362)‡	86.4 (28.2) (107/253)‡	77.8 (24.3) (59/149)‡

Results expressed as mean (standard deviation) | **P < 0.01; ***P < 0.001 (compared to preceding annual timepoint) | †People with type 2 diabetes (n = 189) | ‡Ratio of participants with available data (numerator) to total number of completers at timepoint (denominator)

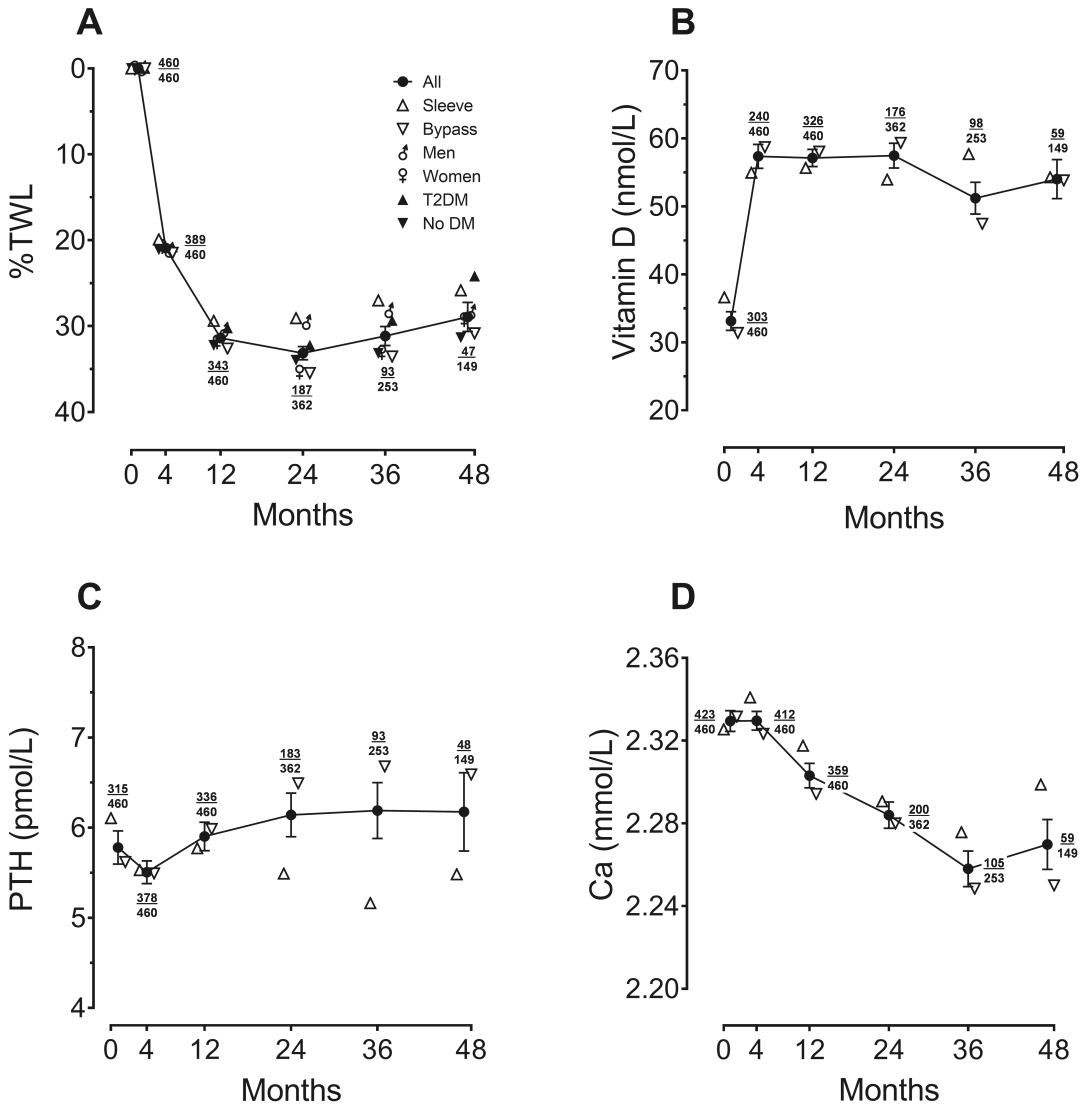

Figure 1. Outcomes of bariatric surgery over 4 years of follow-up: [A] percent total weight loss (%TWL); [B] vitamin D; [C] parathyroid hormone (PTH); and [D] adjusted calcium.
Ratios depict number of participants with available data (numerator) to total number of completers at timepoint (denominator).

Out of 189 people with pre-existing T2DM, 180 had baseline HbA1c recorded; of these, 69.4% had HbA1c ≥ 48 mmol/mol prior to surgery, but by 4 months this prevalence had fallen to 23.3% (P <0.001). At baseline, the mean HbA1c in the gastric bypass group (n = 129) vs. sleeve gastrectomy group (n = 51) was 63.0 vs. 58.2 mmol/mol (ns). At baseline, the mean HbA1c in women (n = 98) vs. men (n = 82) was 60.9 vs. 62.5 mmol/mol (ns). Postoperative reductions in HbA1c were similar with no significant difference between surgical or sex groups.

Vitamin D, parathyroid hormone and calcium

Preoperatively, mean (SD) vitamin D level was 33.1 (23.9) nmol/L (**Figure 1 B**); postoperatively, the vitamin D level increased to 57.4 (27.4) nmol/L at 4 months ($P < 0.001$) and was maintained at subsequent timepoints. There was no significant difference in vitamin D levels between gastric bypass and sleeve gastrectomy throughout the period of study. There was no significant change in mean parathyroid hormone levels overall or by type of surgery throughout (**Figure 1 C**). Mean adjusted calcium levels reduced significantly by 12 months post-surgery and beyond compared to baseline (**Figure 1 D**).

At baseline, there was a significant inverse correlation between BMI and vitamin D level ($r = -0.270$, $p < 0.001$) (**Figure 2 A**). This inverse correlation became weaker with significant weight loss at 12 months ($r = -0.130$, $P < 0.02$) and thereafter. This was corroborated by a significant positive correlation between %TWL and vitamin D level i.e. the greater the excess weight loss, the higher the vitamin D level. At 4 months, a significant positive correlation between %TWL and vitamin D was demonstrated ($r = 0.142$, $P < 0.034$). This was also seen at 12 months ($r = 0.113$, $P < 0.049$) and 24 months ($r = 0.185$, $P < 0.019$) but not thereafter. However, at no timepoints was there a significant correlation between %TWL and change in vitamin D level from baseline**.**

At baseline there was an inverse correlation between vitamin D and PTH levels ($r = -0.204$, $P < 0.001$) (**Figure 2 B**). A similar strength of correlation was seen at all timepoints from 4 to 48 months post-bariatric surgery. There was positive correlation between vitamin D and adjusted calcium levels at baseline ($r = 0.144$, $P = 0.013$) (**Figure 2 C**), and at 12 months post-bariatric surgery ($r = 0.161$, $P < 0.004$) but not thereafter. PTH and adjusted calcium levels were inversely correlated at baseline ($r = -0.142$, $P = 0.012$) (**Figure 2 D**), and at 12 months post-bariatric surgery ($r = -0.160$, $P < 0.003$).

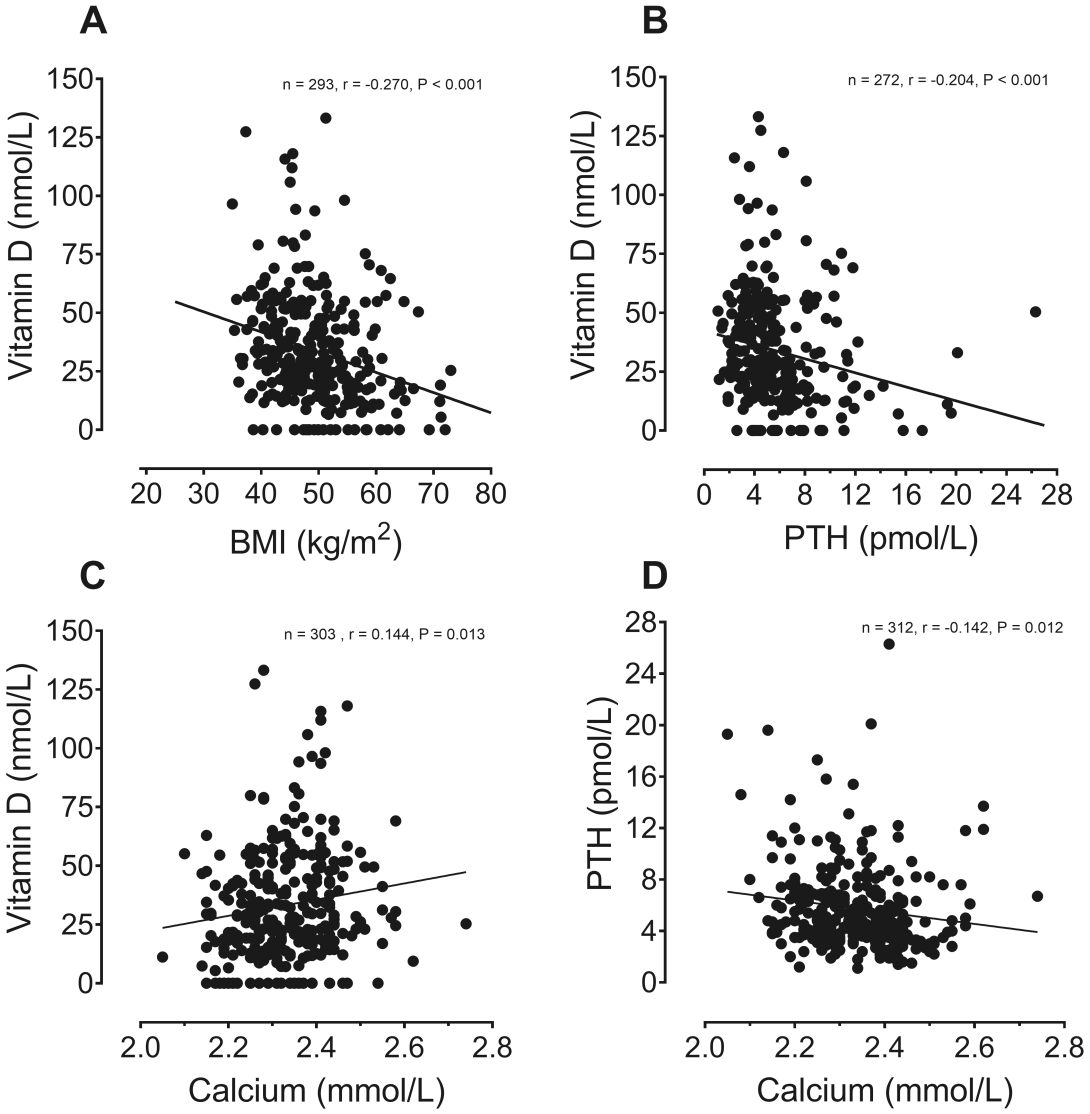

Figure 2. Correlation plots at baseline: [A] vitamin D vs. body mass index (BMI); [B] vitamin D vs. parathyroid hormone (PTH); [C] vitamin D vs. adjusted calcium; [D] PTH vs. adjusted calcium.

At baseline, 43.2% of all patients had vitamin D deficiency and 34.7% had insufficiency; rates of vitamin D sufficiency increased significantly at all post-operative timepoints, with no significant differences between procedures or sexes (**Figure 3 A**). Patients with high PTH or low adjusted calcium levels had higher rates of vitamin D insufficiency/deficiency (**Figure 3 B**).

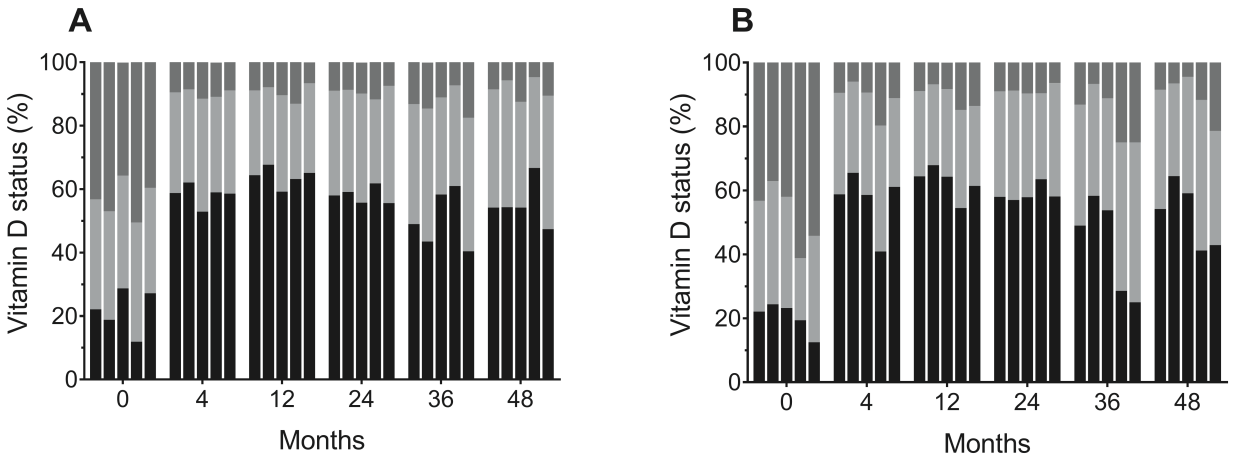

Figure 3. Vitamin D status: proportions of patients with vitamin D sufficiency (>50 nmol/L; bottom stack, black), insufficiency (25–50 nmol/L; middle stack, light gray) or deficiency (<25 nmol/L; top stack, dark gray); [A] in each set, the first column depicts all patients, second column gastric bypass, third sleeve gastrectomy, fourth men and fifth women; [B] in each set, the first column depicts all patients, second column patients with normal PTH, third patients with normal adjusted calcium, fourth patients with high PTH and fifth patients with low adjusted calcium levels.

At baseline, 23.8% of all patients had raised PTH levels; rates of hyperparathyroidism increased over time but there were no significant differences between procedures or sexes (**Figure 4 A**). Among patients with vitamin D sufficiency, 20.0% had raised parathyroid hormone levels at baseline and hyperparathyroidism persisted at rates of 15–30% throughout the 4 years of follow-up (non-significant change compared to baseline at all timepoints), whilst patients with vitamin D insufficiency/deficiency or hypocalcaemia had higher rates of hyperparathyroidism **(Figure 4 B**).

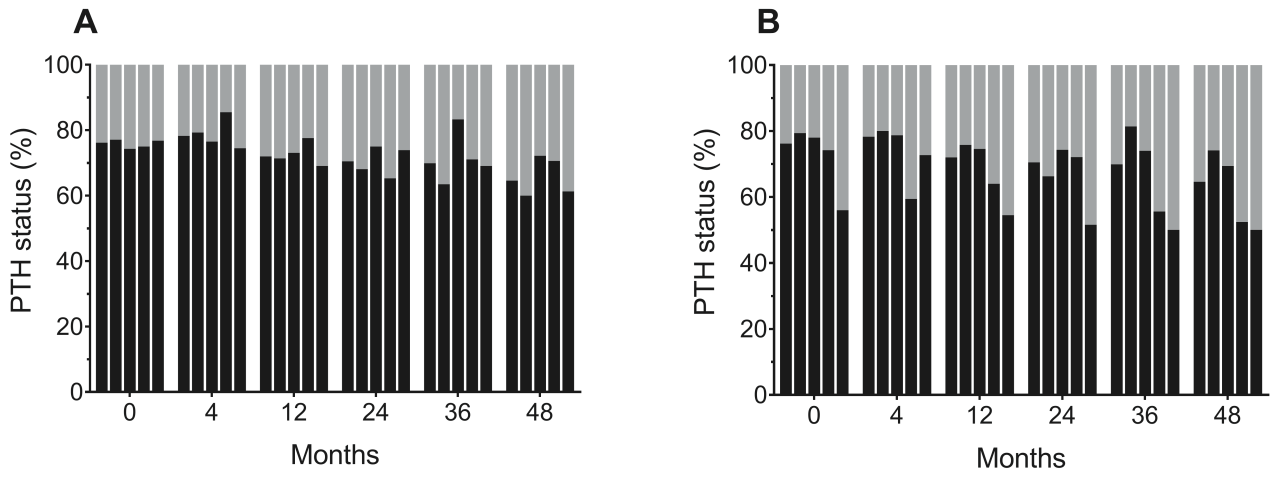

Figure 4. Parathyroid hormone (PTH) status: proportions of patients with normal PTH (bottom stack; black) or high PTH (top stack; light gray); [A] in each set, the first column depicts all patients, second column gastric bypass, third sleeve gastrectomy, fourth men and fifth women; [B] in each set, the first column depicts all patients, second column patients with vitamin D sufficiency (> 50 nmol/L), third patients with normal adjusted calcium, fourth patients with vitamin D insufficiency/deficiency (≤ 50 nmol/L) and fifth patients with low adjusted calcium levels.

At baseline, 8.5% of all patients had low adjusted calcium levels; rates of hypocalcaemia increased over time, with no significant differences between procedures or sexes (**Figure 5 A**). Patients with hyperparathyroidism or vitamin D insufficiency/deficiency had higher rates of hypocalcaemia (**Figure 5 B**).

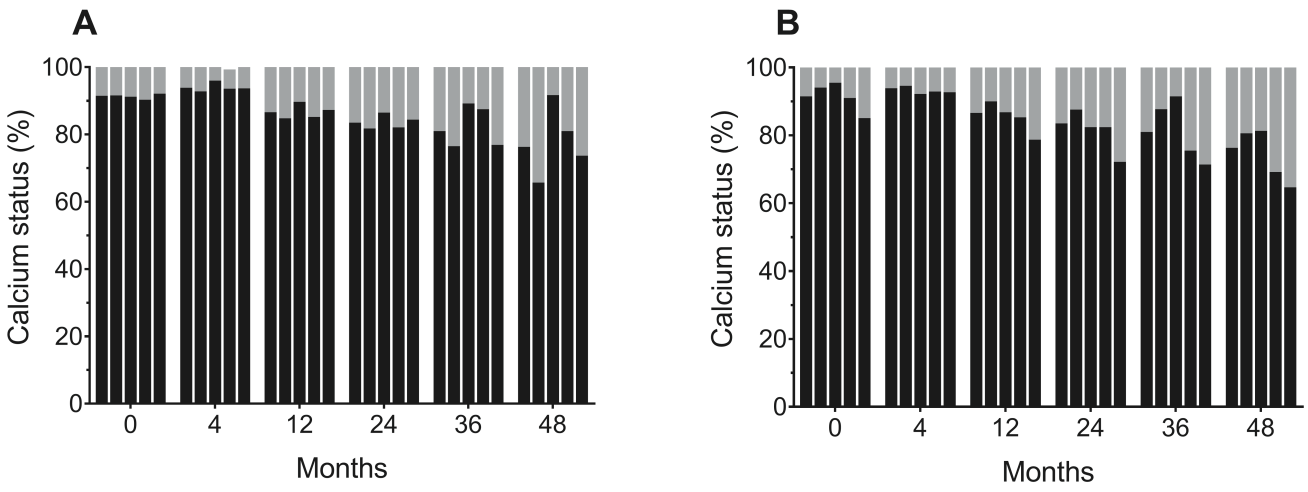

Figure 5. Calcium status: proportions of patients with normal adjusted calcium (bottom stack, black) or low adjusted calcium (top stack, light gray); [A] in each set, the first column depicts all patients, second column gastric bypass, third sleeve gastrectomy, fourth men and fifth women; [B] in each set, the first column depicts all patients, second column patients with normal PTH, third patients with vitamin D sufficiency (> 50 nmol/L), fourth patients with high PTH and fifth patients with vitamin D insufficiency/deficiency (≤ 50 nmol/L).

Discussion

We assessed longitudinal vitamin D status in patients undergoing bariatric surgery. The pre-operative mean vitamin D level of 33 nmol/L with over two-fifths of patients having deficiency and a further one-third having insufficiency was not dissimilar to previous studies (10). Patients in our centre were routinely advised daily vitamin D supplementation ≥ 800 IU. We observed significant improvement in vitamin D level postoperatively, with no differences between types of surgery, that was maintained over 4 years of follow-up. However, some patients remained deficient in vitamin D despite supplementation, consistent with previous research (7,10,11,13,15,24–27). Previous meta-analyses have reported wide variation in vitamin D supplementation regimens with a proportionate increase in postoperative vitamin D levels (10); whilst a daily vitamin D supplement of more than 800 IU significantly reduced the prevalence of vitamin D depletion at 12 months, a dosage of less than 800 IU did not (19).

Vitamin D deficiency has a significant background prevalence in the UK, with 17% of adults aged 19-64 having levels lower than 25 nmol/L (28,29). Rates of deficiency increase to 30-40% during winter months as most vitamin D is obtained through the action of sunlight on the skin. This is influenced by many factors including, but not limited to, season, use of sunscreen, cloud cover, time spent outside during the optimal latitude of the sun and amount of skin exposure. The reference nutrient intake (RNI) for Vitamin D is 10 microgram per day (29). Dietary sources of vitamin D include oily fish, eggs, meat, fortified cereals and fat spreads, and nutritional supplements. Eating a diet rich in Vitamin D will help to prevent deficiency, but data from the national diet and nutrition survey show that adults from the UK aged 19-64 have an average daily intake of 42% of the RNI from diet and supplements (28). Therefore, the advice for the general population is to routinely take a supplement to ensure healthy vitamin D status, especially during the autumn and winter months (30).

It has been consistently demonstrated that there is a high prevalence of vitamin D deficiency in patients with obesity. One meta-analysis reported vitamin D deficiency to be 35% more prevalent in individuals with obesity than in controls of normal weight, and 24% more than in those who were classed as overweight (27). Several different reasons have been proposed. Evidence suggests that vitamin D becomes sequestered in adipose tissue in individuals with obesity, contributing to low circulating levels of vitamin D. One study found visceral fat (which individuals with obesity have a high proportion of) to have 21% higher vitamin D levels than subcutaneous fat (31). Oral vitamin D may also be reduced in individuals with obesity due to low intake of vitamin D-containing foods – termed high-calorie malnutrition. Individuals with obesity may also have less exposure to sunlight due to a relatively sedentary lifestyle, resulting in less vitamin D being produced in the skin. It has also been suggested that obesity-related steatosis of the liver may lead to reduced activation of vitamin D (32). Recent research has found that the proinflammatory state which comes with obesity may lead to alterations in vitamin D metabolism and lowered circulating levels (33). Furthermore, chronic kidney disease which is more prevalent in obese individuals (34), is associated with vitamin D deficiency (35). Adiposity and BMI have been shown to have a negative correlation with serum Vitamin D levels (16,36–38). Thus, bariatric surgical candidates have a predisposition to vitamin D deficiency. Studies have reported that typically over 50%

of bariatric surgical candidates are deficient in vitamin D, and in some cases over 90% (26).

There have been concerns that bariatric surgery may induce or exacerbate vitamin D deficiency. Key reasons for this which have been proposed include reduced oral intake and malabsorptive effects of the procedures. For these reasons, routine supplementation is recommended after bariatric surgery, though there is no consensus on dose or regimen, with doses used in studies ranging from 200 – 28,500 IU daily (10). In the USA, consensus guidelines recommend at least 3000 IU daily (39). The British Obesity & Metabolic Surgery Society recognises that usual practice following gastric bypass or sleeve gastrectomy is oral supplementation with 20 micrograms (800 IU) vitamin D (commonly as a combined calcium and vitamin D tablet) per day, with higher doses if still deficient (8). Most studies have reported difficulty in optimisation of vitamin D levels after bariatric surgery, despite varying doses of supplementation. Reasons for this may include malabsorption of supplements, inadequate supplement dose and lack of concordance. Residual excess adiposity may also play a role. Some studies have suggested that vitamin D optimisation is possible in many patients after bariatric surgery. In particular, a rapid rise in vitamin D has been demonstrated in the period immediately after surgery (10,19). This may be due to a high level of compliance with supplementation soon after surgery. It has also been suggested that the rapid weight loss following surgery may liberate vitamin D sequestered in visceral fat, augmenting vitamin D levels initially (40).

Previous studies of vitamin D status after bariatric surgery have illustrated a marked variation in numerous factors, most notably vitamin D dose and regimen, definition of vitamin D deficiency and length of follow-up. However, some authors have performed systematic reviews and meta-analyses, and have reported a variety of findings (10,15,19,24–26,41,42). Most studies have only followed patients for 1 or 2 years post-operatively, limiting their long-term significance. Some have found postoperative supplementation to be ineffective almost universally while others have found some regimens to be successful in preventing post-operative vitamin D deficiency in some patients (10,19,25,26,41,42). Many have reported that requirements for vitamin D supplementation vary significantly between patients. It is our policy to screen all candidates prior to their operation and treat deficiencies. Our centre recommends maintenance supplementation with 1000–1200 mg of elemental

calcium combined with vitamin D > 800 IU daily. We have found that this regimen with subsequent monitoring and dose modification can prevent vitamin D deficiency postoperatively in most patients. We found no significant difference in vitamin D deficiency rates between gastric bypass and sleeve gastrectomy. Secondary hyperparathyroidism was seen in up to two-thirds of patients with vitamin D deficiency. Intriguingly, about one-fifths of patients with vitamin D sufficiency also had raised PTH levels. Despite calcium supplementation and maintenance of normocalcaemia, notably, there was a statistically significant fall in mean calcium levels following bariatric surgery by 12 months and beyond. It has been suggested that deficiency in calcium seems to play the driving force for secondary hyperparathyroidism in patients with vitamin D sufficiency (14); serum calcium levels may be maintained within the normal range by bone resorption in these patients. Clinically significant reduction in bone mineral density (BMD) has been reported in three-fifths of patients at 6 months and in seven-tenths at 12 months after bariatric surgery, alongside a significant association with greater weight loss (18). Individuals with T2DM treated by gastric bypass, compared to individuals with T2DM of similar age and body composition not treated by gastric bypass, have been reported to have lower BMD, lower bone strength, and increased levels of several bone turnover markers (43). Another recent study has also reported that levels of bone turnover markers increased significantly after gastric bypass surgery (44). Systematic vitamin D and calcium supplementation seems advisable although its beneficial effect on BMD in the post-surgical population remains to be seen.

Limitations

Despite careful manual collection of data from a prospective electronic database, our study was restricted by the retrospective nature of the cohort analysis. Whilst we did not record racial demographics, we should remark that our catchment population is comprised of mostly White British ethnicity. In common with many longitudinal observational studies, data attrition due to patients being lost to follow-up was a major limitation of this study. Despite a large initial sample size, data for key measurements (such as vitamin D) was unavailable for 30% of completers at 1 year and 60% of completers at 4 years. We were unable to collect data on personalised

dose adjustments of supplements and patients' adherence to treatment, or seasonal variations in vitamin D levels. Although vitamin D, calcium and PTH are useful indicators, we were unable to include markers of bone turnover, bone densitometry or fracture data and so cannot report directly the skeletal effects of bariatric surgery in our cohort. Despite these limitations, our observational study confirms that routine supplementation along with rigorous monitoring can prevent vitamin D deficiency after bariatric surgery in most patients.

Conclusion

We report that vitamin D deficiency was prevalent in bariatric surgical candidates pre-operatively and routine vitamin D supplementation can prevent post-operative vitamin D deficiency in most patients undergoing weight loss surgery. However, some patients remain poorly optimised despite this. Further research could investigate risk factors for difficult-to-optimise vitamin D status as well as different supplementation methods and dose regimens.

a) Conflict of Interest Statement

All authors declare no conflicts of interest.

b) Ethical Approval Statement

For this type of study formal consent was not required.

c) Informed Consent Statement

Does not apply

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Chapter 10: Discussion

10.1 Introduction

Current evidence suggests that obesity itself is a risk factor for CVD and metabolic disease (1). The metabolic syndrome is common in obesity and is associated with increases in CVD risk (2). It is still inadequately comprehended how the different facets of the metabolic syndrome i) interact with each other and ii) augment CVD risk. This is because it is difficult to study different components of this complex syndrome prospectively over decades, their interaction with each other and how they increase CVD risk when clustered together. Bariatric surgery corrects different aspects of the metabolic syndrome simultaneously over a relatively short period, particularly insulin resistance and T2DM (3-6). It therefore presents a unique model by which to further study the inter-related dependence of individual factors and provide an insight into the compound and multimodal nature of CVD risk in the metabolic syndrome which Reaven had postulated was driven by hyperinsulinaemia (7-10). Furthermore, other mechanisms by which CVD risk are increased are still not fully understood. In recent years, more novel risk factors for CVD are gaining importance, including impairment of HDL functionality and the presence of anti-apoA-1 IgG antibodies (11-14). This is especially pertinent because an elegant large-scale analysis by Lu *et al.* showed that only 46% of coronary artery disease and 76% of stroke were explained by the traditional reversible risk factors of hypertension, raised cholesterol and hyperglycaemia in people with a raised BMI (15).

Microvascular disease is a major source of morbidity in obese patients with T2DM. Obesity has been shown to increase the incidence of retinopathy, nephropathy and neuropathy (16-18). There is still little evidence relating to the effect of bariatric surgery on the microvascular complications of T2DM, particularly so for neuropathy. Previous studies have shown a reduced incidence of microvascular disease in obese people following bariatric surgery although this has, to date, simply been an observation without there being any robust evidence as to what the protective factors may be (19, 20).

Another source of distress in obese people is sexual dysfunction (21, 22). Obesity related erectile dysfunction in men is relatively common yet the cause of this has

been poorly understood (21, 22). Theoretically, amongst the main factors that would influence obesity-related sexual dysfunction in men would be hormonal factors (testosterone) (23, 24), vascular factors and peripheral neuropathy which has an increased prevalence in people with obesity (18). The role of small fibre neuropathy in the pathogenesis of erectile dysfunction has remained underinvestigated.

Bariatric surgery, although protective against most metabolic disease, has also been implicated in secondary hyperparathyroidism and potential unfavourable effects on bone health (25-27). Vitamin D deficiency in obesity is common (28, 29). In patients who have had metabolic surgery, the risk of worsening vitamin D deficiency (amongst other potential nutritional deficit), is of specific concern with regards to bone health (29, 30). Existing clinical guidelines do promote routine supplementation of vitamin D post-bariatric surgery (31). However, it remains unclear whether supplementary oral vitamin D does indeed protect against vitamin D deficiency in the unique setting of patients following bariatric surgery (considering their post-operative anatomical distortions).

10.2: Bariatric Surgery as a model to explore the basis and consequences of the Reaven hypothesis: Small, dense low-density lipoprotein and interleukin-6 (Chapter 4).

Gerald Reaven had described a conglomerate of excessive insulin levels/insulin resistance, central obesity, hypertriglyceridaemia, impaired glucose tolerance, changes in inflammatory cytokines and reductions in HDL-C (7, 8). Following on from this work, sdLDL was found and this subtype of LDL possibly carries substantial atherogenicity as it is prone to both glycation and oxidation (32-36). In this study, we assessed the relationships between the different aspects of the Reaven hypothesis by using bariatric surgery as a model due to its ability to markedly reduce levels of insulin and insulin resistance. We also measured the effect of bariatric surgery on atherogenic lipoproteins and inflammatory cytokines which could augment CVD risk. We showed that following bariatric surgery induced weight loss by obese persons, along with the improvements in hyperinsulinaemia and insulin resistance (HOMA-IR), there were associated changes in triglycerides, HDL-C and hsCRP, all of which

Reaven had clustered together as associated factors (7). Bariatric surgery therefore did provide an ideal model in which to tie together the associations that Reaven had originally postulated. Furthermore, we also demonstrated significant reductions in sdLDL and IL-6 and these changes happened independent of statin treatment and T2DM. The related reductions in sdLDL and IL-6 post-bariatric surgery may also offer an insight into how the metabolic syndrome causes a predisposition to atherosclerosis and indeed how bariatric surgery, by improving the metabolic syndrome, reduces the incidence in CVD (as observed in longitudinal studies).

10.3: Enhancements in High-Density Lipoprotein Functionality after Bariatric Surgery are related to reductions in adipose tissue and systemic inflammatory cytokines (Chapter 5)

Epidemiological studies have shown an association between low HDL-C and CVD (37). However, in four randomised trials, pharmacological enhancement of HDL-C, using CETP inhibitors and nicotinic acid, did not confer protection against CVD (38-42). In the REVEAL study the improvement in CVD risk was related to a decrease in apoB rather than the increase in HDL-C despite the fact that anacetrapib, dalcetrapib and evacitrapib increase CEC *in vitro* albiet, that of dalcitrapib was genotype dependant (43-45). HDL has a number of functions that protect against CVD, of which RCT, the pathway of removing excess cholesterol is a fundamental one (46, 47). Moreover, large clinical trials have shown an association between reductions in CEC, a component of the RCT pathway, and CVD (11, 12). However, the currently established method of measuring CEC *in vitro* is using commercially available cells. This therefore only provides a functional estimate of different components of RCT and robustness of the system *in vivo*, which consists of both active and passive transport (11). The RCT pathway in turn also depends on a capacity of peripheral cells to transport cholesterol out and HDL to accept any cholesterol that is effluxed. Adipose tissue is an accessible tissue site in which there is marked lipid exchange and therefore provides an ideal area to study changes in the key transporters involved in RCT and to understand what drives changes after an intervention. In addition, PON1 is an HDL related enzyme that mediates its antioxidant function (47)

and it has been shown to predict CVD risk (48-50) and therefore has the potential to be a therapeutic target.

In this study the key aim was to establish if there is a change in HDL functionality (defined by changes to the key components of RCT, HDL's CEC and quantification of the transport channels [ABCG1, ABCA1 and SR-B1] involved, and PON1 activity) after bariatric surgery. In addition, an understanding of the factors that affected HDL functionality was sought by examining changes systemically and in adipose tissue. There were significant improvements in CEC, PON1 activity and ABCG1 gene expression (in adipose tissue) following bariatric surgery implying an enhanced post-operative functioning of HDL and this is a novel finding. Furthermore, for the first time, this study also displayed that there was an inverse correlation between CEC and post-operative TNF-α in serum as well as between ABCG1 gene expression (fold change) and the histological change in TNF-α immunostaining in adipose tissue. Regression analysis also showed that percentage changes in TNF-α were an independent predictor (negative) of percentage changes in CEC, another novel finding. The changes in CEC although independent of HDL-C and BMI changes, were related to reductions in hyperglycaemia after bariatric surgery, a fourth novel finding. Furthermore, the study showed marked reductions in BMI, markers of inflammation (hsCRP), insulin resistance (HOMA-IR), dysglycaemia (HbA1c) and oxidative stress (MPO mass) implying a plethora of concurrent CVD protective effects of bariatric surgery.

10.4: Bariatric Surgery Leads to a Reduction in Antibodies to Apolipoprotein A-1: A Prospective Cohort Study (Chapter 6)

There is a growing interest in anti-apoA-1 IgG as a non-traditional CV risk factor with it being established as an independent predictor of incident CVD in the general population (51) as well as among patients with underlying inflammatory conditions (52). With chronic low-grade inflammation being a hallmark of obesity and the well-established link with CVD, we investigated the presence of anti-apoA-1 IgG in a cohort with severe obesity and the changes following bariatric surgery.

In this study we showed a marked reduction in anti-apoA-1 IgG levels as early as 6 months after bariatric surgery. Furthermore, there was a significant reduction in the proportion of patients with anti-apoA-1 antibody seropositivity following bariatric surgery. We also found that patients with antibody seronegativity 12 months after bariatric surgery accrued a greater %EBMIL. Indeed, in a binary logistic regression model, the anti-apoA-1 IgG titre at twelve months post-operatively was shown to be a significant predictor (inverse association) of the ability to achieve a %EBMIL of >50%, a key measure of surgical success (53).

We found a higher prevalence of autoantibody positivity in our cohort compared to general population of relatively high prevalence of CVD (54), as well as high CV risk cohorts such as patients with end-stage renal failure on dialysis (55). This was also two-fold higher than a population with severe obesity but no history of metabolic complications (56), suggesting a heterogeneity in CV risk even among the severely obese population.

The prevalence of CVD was higher among our anti-apoA-1 IgG positive cohort which is in support of the role of anti-apoA-1 IgG in the pathophysiology of CVD in obesity (57).

This inferred CV risk relating to anti-apoA-1 IgG, however, may potentially be modifiable by an intervention such as bariatric surgery. Further longitudinal studies with CV outcome measures, however, are required to clarify if the alteration in autoantibody level and status is reflected in improvements CV risk.

In the study we could not determine the mechanisms behind the reductions in anti-apoA-1 IgG levels, changes in antibody status or how anti-apoA-1 potentially affects %EBMIL. An understanding of the means by which changes in anti-apoA-1 antibodies are affected following bariatric surgery will require a specifically designed study to undertake such analysis. The relationship between anti-apoA-1 antibodies and %EBMIL also requires further interrogation in a larger cohort.

10.5: Improvements in Diabetic Neuropathy and Nephropathy after Bariatric Surgery: A Prospective Cohort Study (Chapter 7)

This study showed that twelve months after bariatric surgery in patients with obesity complicated by T2DM, there were significant improvements in DKD and DN without any changes in DR. There were also reductions in BMI and blood pressure, similar to other studies (6, 58). HbA1c was also reduced post-operatively resulting in a 77% remission rate from T2DM. There were significant improvements in both triglyceride levels as well as HDL-C. The improvements in DN were manifest by improvements in the NSP, CNBD, CNFD and CNFL. The improvements in CNFL were inversely associated with changes in triglycerides which supports previous studies which showed a relationship between triglyceride levels and markers of small-fibre neuropathy (59). However, this is the first study to show an association between an intervention that reduces triglycerides correspondingly improving CNFL. There was a significant reduction in serum creatinine and eGFR (which was excessively high at baseline) which resulted in a decrease in the proportion of patients who met the criteria for glomerular hyperfiltration. At baseline, there was little evidence of clinically relevant proteinuria in the cohort and post-operatively although there was a reduction in uACR, this was not statistically significant. Both changes in the systolic blood pressure and %EBMIL were seen to influence the changes in eGFR post-operatively. The cohort in this study had a relatively short average duration of diabetes with optimal HbA1c levels pre-operatively. Consequently, there was a low prevalence of overt microvascular disease and the sensitivity of our methods therefore allowed for detection of early or even subclinical disease. Bariatric surgery which allows for aggressive management of different metabolic risk factors, in this study, has been shown to improve early signs of microvascular complications. This may contribute to previous longitudinal observations from large cohorts which showed a reduction in the incidence of microvascular complications of T2DM post-bariatric surgery.

10.6: Male sexual dysfunction in obesity: The role of sex hormones and small fibre neuropathy (Chapter 8)

Erectile dysfunction reflects a complex underlying disease process and, in the presence of diabetes and obesity, can be a manifestation of microvascular disease. With hypogonadism being common in obesity and diabetes, testosterone replacement is often the focus in clinical practice despite an inconsistent body of evidence for its benefits. In our study, we simultaneously assessed the relationship between sexual and erectile dysfunction, peripheral nerve parameters, and testosterone levels in a cohort of patients with obesity and diabetes. We demonstrated an association between small fibre neuropathy and erectile dysfunction, adding support to the central role of small nerve fibre in erectile function. This is also in keeping with the finding of autonomic dysfunction among patients with erectile dysfunction within our study cohort. There was a lack of association between testosterone level and erectile function which suggests that hypogonadism may not be a major factor for presence of erectile dysfunction in obesity, adding to the uncertainty on the benefits of normalising testosterone levels in these patients. The cross-sectional design of this study limits our ability to draw conclusions on cause and effect. Given the positive effect of bariatric surgery on small fibre neuropathy we have demonstrated earlier, a prospective study assessing the impact of surgery-induced improvement in small nerve fibre parameters on erectile function would provide further insight to the role of small nerve fibre in obesity and diabetes.

10.8: Vitamin D status after gastric bypass or sleeve gastrectomy over 4 years of follow-up (Chapter 9)

Previous studies have shown an association between bariatric surgery and impairment of bone health with the assertion that this may be due to metabolic derangements, including vitamin D deficiency (25). Furthermore, vitamin D deficiency in obesity and after bariatric surgery has been found to be relatively common (30). In this retrospective study we tested whether routine vitamin D replacement (as advocated by United Kingdom national clinical guidelines (31)) in

patients who underwent bariatric surgery would be effective in reducing vitamin D deficiency over a follow-up period between 12 to 48 months post-surgery.
We found that daily calcium and vitamin D supplementation prevented vitamin D deficiency post-operatively. Indeed, with supplementation, there was a reduction in the existing prevalence of vitamin D deficiency post-operatively that was seen in obese patients before surgery. There was no observable procedure-specific or gender effect on post-operative vitamin D levels. We also found a relationship between serum vitamin D levels and BMI, both pre- and post-operatively.
Although the markers of bone health were assessed in a longitudinal fashion (retrospectively) we did not relate this to fractures, changes in bone mineral density or bone turnover markers. Further studies will need to assess if, as we have shown, maintaining sufficient vitamin D levels with supplementation, can reduce adverse bone effects in the long-term.

10.9 Limitations

There are some important limitations. Firstly, the relatively small sample sizes in chapter 4, 5, 6, 7 and 8 did not allow for a comprehensive analysis of mechanistic factors that would have influenced the observations in those studies. However, each of these studies was sufficiently powered to answer the primary aim. The observational nature of the data in all of the chapters does not allow for elimination of confounders. Chapters 4,5,6,7 and 9 did not have a control group either undergoing routine care or having an alternate intervention and so it is difficult to fully quantify the sole effect of bariatric surgery. However, other studies with control groups and meta-analyses have shown bariatric surgery to be superior at achieving beneficial metabolic outcomes. Therefore, it is likely that any lifestyle or pharmacological intervention is unlikely to match the efficacy of bariatric surgery.

The control group in chapter 5 had a mean BMI in the overweight range however this matched the average adult BMI in the United Kingdom and is therefore likely to show clinically meaningful improvements in outcomes. Chapter 9 was a retrospective study and although the data was all collected prospectively, the breadth and accuracy of data collection was therefore limited. There was attrition of data in the study represented in chapter 9; 60% of the participants did not complete the full 4 years of the follow-up period.

Apart from chapters 7 and 8, the studies examined risk factors and due to the follow-up period, the effect of these risk factors translating into adverse clinical outcomes could not be determined. However, such an analysis would need a longitudinal study which is likely to need a follow-up period of at least 5 years. Most of the studies had a predominant female participation which limits the generalisability of findings. However, more females do undergo bariatric surgery, so this is representative of historical and current trends. In addition, there is no robust evidence to suggest that there is a sex-specific effect on outcomes following bariatric surgery.

10.11 Future Work

The work in this thesis has shown that obesity is associated with an impaired metabolic profile and this is reversible by bariatric surgery. There are other factors that have not been analysed. For example, although HDL functionality was assessed in this thesis, the effects of bariatric surgery on atherogenic modifications of LDL are largely unknown and therefore require further study.

The observation that anti-apoA-1 IgG can potentially affect BMI outcomes following bariatric surgery will need to be examined further and if indeed, this finding is validated in a larger cohort, the reasons behind such a relationship need to be established.

The findings of significant improvements in neuropathy in people with T2DM requires further research to identify any mechanisms that would offer a therapeutic target. It would also be of value to assess if bariatric surgery has a similar effect on neuropathic measures in a cohort with non-diabetic obesity related neuropathy.

Longitudinal studies analysing the specific effect of the risk factor reduction seen in the studies included in this thesis are imperative to understand if these relatively short-term improvements lead to long-term benefit. It is important to link the changes seen in the post-operative state to reductions in CVD, microvascular disease and osteoporosis and fractures.

The effect of bariatric surgery on sexual dysfunction in men needs further study to consolidate the observations seen in chapter 8, especially focusing on which factors, if improved, can treat sexual dysfunction.

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Appendix

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Manchester University NHS Foundation Trust

Study Title

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

- You are being invited to take part in a research study to look at cholesterol (fat) metabolism and cardiovascular health.
- This sheet provides you with the information about the study and how it involves you.
- Before you decide it is important for you to understand why the research is being done and what it will involve.
- Please take time to read the following information carefully before deciding on whether to take part or not.

Introduction

Apart from weight loss, bariatric surgery is thought to have a major impact on fat metabolism and nerve function. Changes to the levels of cholesterol (a type of fat) in the bloodstream have profound effects on the health of the heart and blood vessels. Some forms of cholesterol are beneficial to the body while others are harmful. The mechanisms by which these effects occur are not clear. One of the enzymes which can increase the effectiveness of the good cholesterol is called Paraoxonase 1 (or PON1).

In this study, we will try to determine which factors affect PON1 activity and nerve and blood vessel function before and after bariatric surgery.

What is the purpose of this study?

The purpose of this study is to relate changes of PON1 activity with function of blood vessels and to evaluate nerve function before and after bariatric surgery. This will

help us to understand why obese individuals with and without diabetes have an increased risk of vascular diseases and how the risk can be reduced. It helps us to understand how weight loss may influence changes in nerves and their structure and function.

Why have I been chosen?

You have been scheduled for bariatric surgery and are therefore suitable for the study. We hope to confirm that weight loss after surgery results in favourable changes to PON1 that in turn are associated with lower cardiovascular risk. We will also examine other changes following bariatric surgery including sexual, blood vessel and nerve function.

What will I have to do if I take part?

If you agree to take part, we will confirm that you have understood the study and that you meet with the study criteria. You will be asked to sign a consent form for the study and you will need to attend the Wellcome Trust Clinical Research Facility (in Manchester) for 3 visits.

Your first visit will be arranged before the bariatric surgery. During this visit, we will review your medical and medication history. You will have a brief physical examination that includes measurements of height, weight, waist circumference and blood pressure. We will perform an ECG test to assess the health of your heart and measure your blood vessel stiffness using non-invasive methods.

You will be asked to attend for all the visits having fasted overnight (for at least 10 hours) so that fasting blood samples can be taken from you. A total of 60ml (about 12 teaspoons) of blood will be taken from you. Most of the blood samples will be analysed in the Manchester or Salford laboratories. A small sample of serum or plasma will be retained for future research at the end of the study. In addition, a small frozen anonymised blood sample will be sent to laboratories in Switzerland or Australia for further tests. The tests to be carried out in these laboratories are for research and not for clinical/ diagnostic purposes. The tests will provide more information on the relationship between fat metabolism and diabetes. Additionally,

we will ask you to provide a sample of saliva so that hormone testing can be carried out which will also help study the link between hormonal status, fat metabolism and diabetes. You will be asked to give a sample of urine and a stool sample for analysis. When the analyses are completed, the samples will be destroyed. We will ask you to complete a sexual function questionnaire.

Nerve Function Tests consist of:

- Short questionnaire on pains (if any) in your legs.
- Nerve conduction study. Nerves in your legs are stimulated resulting in momentary muscle twitching. This may cause minor fleeting discomfort. Your ability to sense different temperatures and vibration in your lower legs will be measured.
- Corneal sensitivity is assessed with an air puff stimulus to the front of your eyes with no direct contact.
- A corneal confocal microscope (CCM) will be used to examine the number of nerves in the front part of the eye. A drop of anaesthetic is applied to numb the front of the eye. This allows a gel on the lens of the camera to touch the front of the eye for 1-2 minutes whilst we record images of the cornea.

We will also ask your permission to obtain photographs of the blood vessels at the back of your eyes. The photographs of the back of your eye will be taken using a special camera that does not require the use of dilating drops. There will be no direct contact of the camera with your eyes when these photos are obtained.

Also during this first visit we will take a small sample of fat from you (fat biopsy; see next section).

Visits 2 and 3 will take place 6 and 12 months after your weight loss surgery. All the measurements will be repeated during these visits, except for the fat biopsy, which will only be repeated at the 6 month visit.

What does the fat biopsy involve?

A small sample of fat and skin is taken from the buttock area. This will be done after numbing the area with a local anaesthetic agent so that it will not hurt. You will have

3-4 stitches at the biopsy site which will be covered with a sterile dressing. The stitches are to be removed 7 to 10 days after the procedure either by your GP or at the Wellcome Trust Clinical Research Facility. Information regarding care of the site will be discussed and a leaflet regarding this will be given to you on the day of the procedure. A small sample of fat will also be also taken from the fat inside your abdomen (visceral fat) during the weight loss surgery while you are under general anaesthesia.

What are the possible risks of taking part?

The blood samples will be taken by an experienced doctor or nurse and the only risk involved may be some bruising at the puncture site. Very rarely there may be a mild infection at the biopsy site. This happens approximately once in every 25 procedures (a rate of 4%). If this occurs, a short course of antibiotics from your GP will resolve it. There are no risks involved in the nerve function tests or in obtaining the eye blood vessel photographs.

Are there any possible benefits?

There are no immediate benefits to you. However, the knowledge gained from this study will help us to develop better tests in assessing nerve damage, blood vessel damage and how both of these are related to weight gain and weight loss. It will improve our knowledge of factors leading to heart disease and develop new therapies to prevent it.

Will I be paid for taking part in the study?

No. But your travel expenses will be reimbursed. You have the option of receiving £30 as a single payment for each visit, or you can be reimbursed at each visit on the production of taxi receipt for attending and we will arrange a taxi (paid for by the research team) for your return after your visit.

Do I have to take part?

No, taking part is entirely voluntary. If you do not wish to take part you do not have to give a reason and in no way will your future treatment be affected.

What will happen to my clinical and personal information?

All the clinical information you provide will be encoded (so that your personal details such as name and address are secure) and stored securely. This information will not be revealed to anyone other than the researchers and your GP if you wish the latter to be informed. We would ask your permission to inform your GP of any clinically relevant abnormalities identified during the study.

Complaints

If you have any concerns about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see below). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or the Patient Advisory Liaison Service (PALS). Details can be obtained from the hospital.

What do I do now?

If you have any questions please contact:

- Dr Safwaan Adam, Dr Jan Ho or Dr Shaishav Dhage, Cardiovascular Research Group, University of Manchester, Core Technology Facility (3rd floor), 46 Grafton Street, Manchester, M13 9NT). Tel: 07723526324.

  Alternatively, you can contact the doctor whose clinics you are attending:

- Dr Handrean Soran, Consultant Physician, Department of Medicine, Manchester Royal Infirmary, Oxford road, Manchester, M13 9WL. Tel: 0161 276 4066 (secretary).

- Mr Basil Ammori, Consultant Surgeon, Department of Surgery, Salford Royal Hospital, Stott Lane, Salford M6 8HD. Tel: 0161 789 7373.

Thank you for taking the time to read this and considering taking part in our research. Please discuss this information with your family, friends or GP, if you wish. You will have at least 24 hours to read this information leaflet. After this time, we will contact you again to see if you are still interested in taking part.

NHS
Manchester University
NHS Foundation Trust

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

To be completed by the patient: **Please initial the boxes**

1. I confirm that I have read and understood the patient information sheet [version 6.0. 03.05.2016] provided for the study and I have had the opportunity to ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS trust or regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records. ☐
4. I agree to serum and plasma being retained and stored as a gift to Manchester University and used for future ethically approved research at the end of the study. ☐
5. I agree to fat biopsy samples being retained and stored as a gift to Manchester University and used for future ethically approved research at the end of the study. ☐
6. I consent to my general practitioner being informed of my participation in the study and of any clinically relevant information. ☐
7. I agree for my anonymised blood samples to be transferred to Australia for research purpose. ☐
8. I give my consent to take part in the above study including:
   a. blood tests ☐
   b. Fat biopsy before and 6 months after bariatric surgery ☐
   c. Stool and urine samples ☐
   d. Nerve function tests ☐

e. Eye blood vessel photography ☐

Name…….Date of Birth…….

Signature…… Date……..

<u>To be completed by the investigator or physician or nurse taking consent</u>:

I confirm that I have fully explained and discussed with the patient the nature and purpose of the above study.

Name…….. Position…… (e.g. Investigator)

Signature…… Date…….

Signature of physician if consent was witnessed by a nurse……

Participant's Full Name: Date of Birth: Date:
Investigator:

Physical Measurements:

Height (cm) (no shoes):	
Weight (Kg) (no shoes):	
Waist (cm):	
Hips (cm):	

Brachial blood pressure – (mmHg):

			Average
Systolic pressure lying:			
Diastolic pressure lying:			
Heart rate lying:			
Systolic pressure standing:			
Diastolic pressure standing:			
Heart rate standing:			

Participant's Full Name: Date of Birth: Date:
Investigator:

Neuropathy Symptom Profile

Symptoms of Weakness

Head and neck:

"Do you experience these symptoms to an abnormal degree? Abnormal is beyond what is normal for you."

	Yes	No
1. Drooping of eyelids	☐	☐
2. Double vision (other than momentary)	☐	☐
3. Weakness in chewing	☐	☐
4. Weakness so you experience difficulty moving food in your mouth	☐	☐
5. Weakness in swallowing (more than occasionally)	☐	☐
6. Other weakness of head and neck	☐	☐

Chest:

"Do you experience these symptoms to an abnormal degree?

	Yes	No
7. Weakness in speaking due to shortness of breath	☐	☐
8. Shortness of breath due to muscle weakness	☐	☐
9. Other weakness of the chest	☐	☐

Upper Limbs:

"Do you experience these symptoms to an abnormal degree in one or both sides of your body? "

	Yes	No
10. Weakness of hands, *e.g*. when handling coins, using a key	☐	☐
11. Weakness when straightening fingers	☐	☐
12. Weakness of fingers when clasping or grasping objects	☐	☐
13. Weakness of the wrists	☐	☐
14. Weakness of shoulders and upper arms (e.g. lift objects from a high shelf, comb hair)	☐	☐
15. Other weakness in upper limbs	☐	☐

Participant's Full Name: Date of Birth: Date:
Investigator:

Neuropathy Symptom Profile / continued

Lower Limbs:

"Do you experience these symptoms to an abnormal degree in one or both sides of your body? "

	Yes	No
16. Weakness of the legs so that you slap your feet in walking or cannot carry your weight on your heels	☐	☐
17. Weakness of the legs so that you cannot walk on your toes or forefoot	☐	☐
18. Weakness of your thighs so that you have difficulty climbing or descending stairs, getting up from a chair, sofa or toilet seat, and in these acts you need to use your arms	☐	☐
19. Other weaknesses of the lower limbs	☐	☐

Sensory Symptoms

"Do you experience these symptoms in one region or over the surface of your body to an abnormal degree? Do not include the brief symptoms of "prickling" or "asleep numbness" and discomfort which come from lying too long on an arm, or sitting or lying too long in one position on a leg."

	Yes	No
20. Decrease (or inability) to feel the surface features, size, shape, or texture of what you touch?	☐	☐

If yes, choose only one:

- ☐ in legs only (inc. feet)
- ☐ in arms only (inc. hands)
- ☐ in legs and arms only
- ☐ in mouth, face, or head only
- ☐ other than any of the above

21. Decreased (or inability) to recognize hot from cold?	☐	☐

If yes, choose only one:

- ☐ in legs only (inc. feet)
- ☐ in arms only (inc. hands)
- ☐ in legs and arms only
- ☐ in mouth, face, or head only
- ☐ other than any of the above

22. Decreased (inability) to feel pain, cuts, bruises, or injuries?	☐	☐

If yes, choose only one:

- ☐ in legs only (inc. feet)
- ☐ in arms only (inc. hands)
- ☐ in legs and arms only
- ☐ in mouth, face, or head only
- ☐ other than any of the above

23. A more or less continuous “dead feeling” like Novocain without prickling (tingling)? **Yes** ☐ **No** ☐

If yes, choose only one:

- in legs only (inc. feet) ☐
- in arms only (inc. hands) ☐
- in legs and arms only ☐
- in mouth, face, or head only ☐
- other than any of the above ☐

24. A more or less continuous “prickling” or “tingling” feeling with or without an asleep dead feeling? ☐ ☐

If yes, choose only one:

- in legs only (inc. feet) ☐
- in arms only (inc. hands) ☐
- in legs and arms only ☐
- in mouth, face, or head only ☐
- other than any of the above ☐

25. Unusual sensitivity or tenderness when regions of the body are touched or when the hands or feet are used in manual activity? **Yes** ☐ **No** ☐

If yes, choose only one:

- in legs only (inc. feet) ☐
- In arms only (inc. hands) ☐
- In legs and arms only ☐
- in mouth, face, or head only ☐
- other than any of the above ☐

Participant's Full Name: Date of Birth: Date:
Investigator:

Neuropathy Symptom Profile / continued

	Yes	No
26. Sharp "jabbing" needle-like pains or pulse of pain (lasting seconds or a minute or two)	☐	☐

If yes, choose only one:

- ☐ in legs only (inc. feet)
- ☐ In arms only (inc. hands)
- ☐ In legs and arms only
- ☐ in mouth, face, or head only
- ☐ other than any of the above

27. Burning discomfort?	☐	☐

If yes, choose only one:

- ☐ in legs only (inc. feet)
- ☐ In arms only (inc. hands)
- ☐ In legs and arms only
- ☐ in mouth, face, or head only
- ☐ other than any of the above

28. Deep aching pain?	☐	☐

If yes, choose only one:

- ☐ in legs only (inc. feet)
- ☐ In arms only (inc. hands)
- ☐ In legs and arms only
- ☐ in mouth, face, or head only
- ☐ other than any of the above

29. Other pain? ☐ ☐

If yes, choose only one:

in legs only (inc. feet) ☐
In arms only (inc. hands) ☐
In legs and arms only ☐
in mouth, face, or head only ☐
other than any of the above ☐

Participant's Full Name: Date of Birth: Date
Investigator:

Neuropathy Symptom Profile / continued
Autonomic Symptoms

"Do you experience these symptoms to an abnormal degree?"

	Yes	No
30. Feel faint or actually faint, which only comes upon sitting or on standing, and which cannot be explained by use of blood pressure medication or psychologic stress (e.g. sight of blood)?	☐	☐
31. Repeated nausea or vomiting of undigested food, especially in the morning, which is not due to known stomach or gallbladder disease?	☐	☐
32. Persistent diarrhoea, especially at night which is not due to irritable bowel, or other bowel disease	☐	☐
33. Loss of bladder control, which is not due to gynaecologic problems in women or prostate problems in men?	☐	☐
34. Loss of rectal control, with soiling which is not due to known rectal disease?	☐	☐
35. Inability in men to have sexual erection which is not due to medication or prostate surgery?	☐	☐
36. Inability in men to have emission of seminal fluid, which is not due to medication or prostate surgery?	☐	☐

37. Dryness of the eyes, which is not due to use of medication or known eye disease? ☐ ☐

38. Dryness of the mouth, which is not due to use of medication or known mouth disease? ☐ ☐

EMAS Sexual Function Questionnaire

Study ID number

Introduction

This booklet contains questions that ask about your sexual function. Some questions may be of a sensitive nature. However, your accurate and considered responses are very important for this research.

As with all other information you give us, your answers will be treated in the strictest confidence and used only for the purposes of this research.

When you have completed this questionnaire, please place it in the envelope provided and seal the envelope. In this way, we can make sure that nobody who knows you, your name or address will be able to see this information ***unless you give your express permission****.*

The research nurse attending to you keeps a blank copy of the questionnaire so that you can refer to it if you need help in answering any questions. This will ensure that the copy of the questionnaire that you are completing remains confidential.

You will find further explanations and instructions at the beginning of each section of the questionnaire.

Version 2
July 2007

Section A

This asks about some general background information

Please tick the ONE statement that best describes your circumstances IN THE LAST 4 WEEKS.

I have been living with my wife	☐	1
I have been cohabitating with my partner	☐	2
I have a sexual partner but we did not live together	☐	3
I did not have a sexual partner	☐	4

If you did NOT have a sexual partner in the last 4 weeks, please skip the next two questions and go straight to Section B.

1 In general, would you say that the health of your partner is:

Excellent	☐	4
Very good	☐	3
Good	☐	2
Fair	☐	1
Poor	☐	0

2 How satisfied have you been with your general (non-sexual) relationship with your partner?

Very satisfied	☐	4
Moderately satisfied	☐	3
About equally satisfied and dissatisfied	☐	2
Moderately dissatisfied	☐	1
Very dissatisfied	☐	0

Section B

This section asks about your sexual drive or sexual desire.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

1 How often did you think about sex? This includes times of just being interested in sex, daydreaming or fantasizing about sex, as well as times when you wanted to have sex.

Not at all	☐	0
Once in the last month	☐	1
2-3 times in the last month	☐	2
Once a week	☐	3
2-3 times a week	☐	4
4-6 times a week	☐	5
Once a day	☐	6
More than once a day	☐	7

2 How would you rate your level of sexual desire?

Very low/none at all		**0**
Low		**1**
Moderate		**2**
High		**3**
Very high		**4**

3 Are you worried or distressed by your current level of sexual drive/desire?

Not at all worried or distressed		**0**
A little bit worried or distressed		**1**
Moderately worried or distressed		**2**
Very worried or distressed		**3**
Extremely worried or distressed		**4**

4 Compared with a year ago, has your sexual drive/desire changed?

Increased a lot		**+2**
Increased moderately		**+1**
Neither increased nor decreased		**0**
Decreased moderately		**-1**
Decreased a lot		**-2**

Section C

This section asks about the frequency of your sexual activities.

If you DID NOT have a sexual partner in the last 4 weeks please skip Questions 5 and 6 and go straight to Question 7.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

5 How many times have you attempted sexual intercourse?

Not at all		**0**
Once in the last month		**1**
2-3 times in the last month		**2**
Once a week		**3**
2-3 times a week		**4**
4-6 times a week		**5**
Once a day		**6**
More than once a day		**7**

6 Apart from when you attempted sexual intercourse, how frequently did you engage in activities such as kissing, fondling, petting etc?

Not at all	☐	0
Once in the last month	☐	1
2-3 times in the last month	☐	2
Once a week	☐	3
2-3 times a week	☐	4
4-6 times a week	☐	5
Once a day	☐	6
More than once a day	☐	7

7 How often did you masturbate?

Not at all	☐	0
Once in the last month	☐	1
2-3 times in the last month	☐	2
Once a week	☐	3
2-3 times a week	☐	4
4-6 times a week	☐	5
Once a day	☐	6
More than once a day	☐	7

8 Are you worried or distressed by the overall frequency of your sexual activities (including intercourse, kissing etc and masturbation)?

Not at all worried or distressed	☐	0	Skip Question 8A and go straight to Question 9
A little bit worried or distressed	☐	1	
Moderately worried or distressed	☐	2	
Very worried or distressed	☐	3	
Extremely worried or distressed	☐	4	

8A If you **ARE** worried or distressed by the current frequency of your sexual activities, do you consider it to be

Too frequent	☐	1
Not frequent enough	☐	2

9 Compared with a year ago, has the overall frequency of your sexual activities changed?

Increased a lot		+2
Increased moderately		+1
Neither increased nor decreased		0
Decreased moderately		-1
Decreased a lot		-2

Section D

This section asks about your ability to have an erection. It is common for men to experience erectile problems. This may mean that one is not always able to get or keep an erection that is rigid enough for satisfactory sexual activity (including sexual intercourse and masturbation).

Please tick the ONE statement or response that best describes you IN THE LAST 4 WEEKS.

10 You are

Always able to get and keep an erection which would be good enough for sexual intercourse		1
Usually able to get and keep an erection which would be good enough for sexual intercourse		2
Sometimes able to get and keep an erection which would be good enough for sexual intercourse		3
Never able to get and keep an erection which would be good enough for sexual intercourse		4

11 Are you worried or distressed by your current ability to have an erection?

Not at all worried or distressed		0
A little bit worried or distressed		1
Moderately worried or distressed		2
Very worried or distressed		3
Extremely worried or distressed		4

12 Compare with a year ago, has your ability to have an erection changed?

Increased a lot		+2
Increased moderately		+1
Neither increased nor decreased		0
Decreased moderately		-1
Decreased a lot		-2

Section E

This section asks about your feelings of orgasm or climax leading to ejaculation of semen in response to any sexual stimulation (including intercourse or masturbation).

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

13 When you had sexual stimulation, how often did you have the feeling of orgasm or climax?

No sexual intercourse/masturbation	☐	**0**
Almost never/never	☐	**1**
A few times (much less than half the time)	☐	**2**
Sometimes (about half the time)	☐	**3**
Most of the time (much more than half the time)	☐	**4**
Almost always/always	☐	**5**

14 How satisfied have you been with your sense of control over the timing of your orgasm? (Not being satisfied with 'timing' can mean either taking too long to climax or climaxing too early in the course of sexual activity)

Extremely **satisfied**	☐	**4**	Skip Question 14A and go straight to Question 15
Highly **satisfied**	☐	**3**	Skip Question 14A and go straight to Question 15
Moderately **satisfied**	☐	**2**	
Slightly **satisfied**	☐	**1**	
Not at all **satisfied**	☐	**0**	

14A If you are not extremely or highly satisfied, do you climax

Too early	☐	**1**
Too late	☐	**2**

15 Are you worried or distressed by your current orgasmic experience?

Not at all worried or distressed	☐	**0**
A little bit worried or distressed	☐	**1**
Moderately worried or distressed	☐	**2**
Very worried or distressed	☐	**3**
Extremely worried or distressed	☐	**4**

16 Compared with a year ago, has the enjoyment of your orgasmic experience changed?

Increased a lot	☐	**+2**
Increased moderately	☐	**+1**
Neither increased nor decreased	☐	**0**
Decreased moderately	☐	**-1**
Decreased a lot	☐	**-2**

Section F

This section asks about your morning erections. Men may awaken with an erection although this can vary from day to day.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

17 How frequently did you awaken with a full erection?

Not at all	☐	**0**
Once in the last month	☐	**1**
2-3 times in the last month	☐	**2**
Once a week	☐	**3**
2-3 times a week	☐	**4**
4-6 times a week	☐	**5**
Once a day	☐	**6**
More than once a day	☐	**7**

18 Are you worried or distressed by the frequency of your morning erections?

Not at all worried or distressed	☐	**0**
A little bit worried or distressed	☐	**1**
Moderately worried or distressed	☐	**2**
Very worried or distressed	☐	**3**
Extremely worried or distressed	☐	**4**

19 Compared with a year ago, has the frequency of your morning erections changed?

Increased a lot	☐	**+2**
Increased moderately	☐	**+1**
Neither increased nor decreased	☐	**0**
Decreased moderately	☐	**-1**
Decreased a lot	☐	**-2**

Section G

Considering the answers you have already given above, we would like to know what you think about the quality of your overall sex life.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

20 How satisfied have you been with your overall sex life?

Very satisfied	☐	**4**
Moderately satisfied	☐	**3**
About equally satisfied and dissatisfied	☐	**2**
Moderately dissatisfied	☐	**1**
Very dissatisfied	☐	**0**

21 How worried or distressed have you been about your overall sex life?

Not at all worried or distressed	☐	**0**
Slightly worried or distressed	☐	**1**
About equally worried/not worried or distressed/not distressed	☐	**2**
Moderately worried or distressed	☐	**3**
Very worried or distressed	☐	**4**

22 Compared with a year ago, has your overall sexual satisfaction changed?

Increased a lot	☐	**+2**
Increased moderately	☐	**+1**
Neither increased nor decreased	☐	**0**
Decreased moderately	☐	**-1**
Decreased a lot	☐	**-2**

Thank you for completing this section of the questionnaire.

Please place this questionnaire in the envelope provided and seal it.