Validation and early qualification of pancreatic fat deposition as an imaging biomarker of pancreatic cancer risk

A thesis submitted to the University of Manchester for the degree of Doctor of Medicine in the Faculty of Biology Medicine and Health

2015

Institute of Cancer Sciences

The School of Medical Sciences

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FINAL WORD COUNT: 45,841

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ABSTRACT

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Degree Title	Doctor of Medicine, Faculty of Medical and Human Sciences
Thesis Title	Validation and early qualification of pancreatic fat deposition as an imaging biomarker of pancreatic cancer risk
Submitted	31st December 2015

Introduction Pancreatic cancer is the 10th most common cause of cancer in the United Kingdom (UK) yet the 5th most common cause of cancer related death. Although excess adiposity, measured as body mass index (BMI), is a risk factor for the development of pancreatic cancer the increase in relative risk is modest. Animal models suggest that the intra-organ deposition of lipids may be more specific to disease risk than anthropometric measurements. There is therefore a need to develop non-invasive methods to quantify intra-pancreatic fat deposition as a potential biomarker for pancreatic cancer predisposition. Cancer Research UK (CRUK) sets out clear guidelines for biomarker discovery and development. Potential biomarkers must go through a process of discovery and assay development followed by qualification.

Methods Three streams of research: (i) Stage-one of the PanORAMA project. Assessment of accuracy through comparison of CS-MR and MRS quantified intra-pancreatic fat with histologically quantified intra-pancreatic fat in 12 patients undergoing pancreatic surgery. (ii) Stage-two of the PanORAMA study. Assessment of precision (reproducibility) and comparison with other anthropometric markers of excess adiposity in healthy volunteers (n=15). Refinement of MRS protocols and repeated assessment of precision in healthy volunteers (n=10). (iii) The Breast Risk Reduction Intermittent Dietary Evaluation 2 (BRRIDE-2) trial. Comparison of the effects of Intermittent Energy Restriction (IER) with Daily Energy Restriction (DER) on intra-pancreatic and intra-hepatic fat stores and metabolic markers of disease risk (n=26).

Results (i) CS-MR and MRS had agreement with histological assessment of intra-pancreatic fat, but correlations were only moderate to good (rho 0.672 and 0.781 respectively). (ii) CS-MR, and after refinement, MRS, have clinically acceptable precision. This study tested this principle in intra-pancreatic fat in healthy volunteers with a range of intra-pancreatic fat consistent with the literature on the healthy population. (iii) I found no differences in reduction in intra-hepatic or intra-pancreatic fat when comparing IER with DER. Overall, I found that significant reductions (mean: 6.5%) in both of these ectopic fat stores could be achieved with eight-weeks of dietary intervention.

Discussion More recent hypotheses on the link between excess adiposity and cancer have focused on the importance of within organ local ectopic fat as an abnormal micro-environment favouring cancer development and progression. Importantly, this hypothesis explains the specificity of epidemiological associations between excess adiposity and cancer risk. The observations that within a given individual, in the presence of short-term weight reduction, there are differential changes in local within organ fats – hepatic fat and pancreatic fat – support the specificity hypothesis. This thesis has put us in position to scale-up and explore the importance of intra-organ fats using non-invasive imaging techniques.

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

ABBREVIATIONS

ADF	Alternate Day Fasting
AJCC	American Joint Committee on Cancer
BMI	Body Mass Index
CI	Confidence Interval
CRUK	Cancer Research United Kingdom
CS-MR	Chemical-Shift Magnetic Resonance
CT	Computerised Tomography
DER	Daily Energy Restriction
ESPAC	European Study Group for Pancreatic Cancer
GWAS	Genome Wide Association Study
HCC	Hepatocellular Carcinoma
HFF	Hepatic Fat Fraction
HOMA	Homeostasis Model Assessment
IER	Intermittent Energy Restriction
MDT	Multidisciplinary Team
MR	Magnetic Resonance
MRS	^{1}H Magnetic Resonance Spectroscopy
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NMGH	North Manchester General Hospital
OGTT	Oral Glucose Tolerance Test
PaC	Pancreatic Cancer
PanIN	Pancreatic Intraepithelial Neoplasia
PAT	Pennine Acute Trust
PFF	Pancreatic Fat Fraction
PDAC	Pancreatic Ductal Adenocarcinoma
ROH	Royal Oldham Hospital
REE	Resting Energy Expenditure
RR	Relative Risk
SAT	Subcutaneous Adipose Tissue
TAG	Triaglycerol
UHSM	University Hospital South Manchester
UK	United Kingdom
US	United States of America
USS	Ultrasound Scanning
WC	Waist Circumference
WHR	Waist-to-Hip Ratio
WMIC	Wolfson Molecular Imaging Centre
VAT	Visceral Adipose Tissue

ACKNOWLEDGEMENTS

And my supervisors:

Andrew Renehan
Professor of cancer studies and surgery, University of Manchester

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1   INTRODUCTION

Preface

The local deposition of adipose tissue within organs, termed ectopic fat, is implicated in cancer risk and found in a number of organs including the pancreas (termed pancreatic steatosis). Obesity, defined as body mass index (BMI) greater than 30kg/m², is a risk factor for the development of pancreatic cancer (PaC) and associated with increasing pancreatic fat deposition. As a potential biomarker for the risk of developing pancreatic cancer (PaC), pancreatic steatosis is unexplored. Cancer Research United Kingdom (CRUK) roadmaps provide a robust framework for the development of predisposition biomarkers.[1] The framework consists of three main phases; rationale; biomarker discovery and development (often collectively termed 'validation'); biomarker qualification. This introduction covers the literature that forms the basis of the proposed research programme; evidence that supports our hypothesis and has informed the choice of imaging biomarker and design of the research programme.

The first section of this literature review details the rationale and hypothesis for exploring pancreatic steatosis as a predisposition biomarker for PaC. The review covers the late presentation and poor survival associated with PaC that necessitates the development of novel predisposition biomarkers in PaC. Next I outline the rationale behind our hypothesis. Firstly, I discuss the epidemiological evidence of a link between excess adiposity (as measured by body mass index [BMI]) and PaC. Secondly, the proposed relevance of different adipose tissue depots to cancer development and why surrogates of adiposity (such as BMI) may not accurately quantify the risk attributable to excess adiposity. The review assesses literature on changes in the local inflammatory cell and cytokine milieu that occur secondary to local adipose tissue deposition. The hypothesised link with PaC development is outlined; excess adiposity causes the deposition of adipose tissue within the pancreas (pancreatic steatosis); this creates a pro-inflammatory state within the pancreas; inflammation is a putative mechanism in the development of PaC. The review demonstrates that the link between pancreatic steatosis and other markers of excess adiposity is an under-researched area. I summarise the findings from genome studies that genetic variance in inflammatory and obesity-related pathways influence pancreatic cancer risk. As an analogous hypothesis, evidence from the endocrine literature of a link between pancreatic steatosis and diabetes mellitus is briefly discussed. Finally, findings from animal models that

evidence my hypothesis are summarised, studies linking excess adiposity, inflammatory pathways and PaC development.

The second part of this review turns to the CRUK biomarker validation phase. To address the question of whether pancreatic steatosis is a predisposition biomarker for pancreatic cancer, we first must develop accurate and reproducible biomarkers for the quantitative and descriptive assessment of pancreatic fat deposition (CRUK roadmap classification; Biomarker Development stage I). The literature on imaging methods for the assessment of pancreatic steatosis is covered. Briefly, the limitations of Computerised Tomography (CT) and Ultrasound Scanning (USS), followed by discussion of the emerging magnetic resonance(MR) imaging modalities, chemical shift magnetic resonance (CS-MR) and ^{1}H magnetic resonance spectroscopy (MRS). These modalities are validated in the measurement of histologically-determined hepatic adipose tissue deposition (hepatic steatosis). Evidence for the use of these modalities to measure pancreatic steatosis is limited, with just six papers evaluating CS-MR and eight papers evaluating MRS at the outset of this thesis, and I conclude that there is need for histological validation.

In the third part of this literature review, I consider the hypothesis that pancreatic fat changes quantitatively (hypothesised reduction in intra-pancreatic fat) in response to a weight reduction intervention (CRUK roadmap classification; CTAAC/BIDD Qualification Stage 1), and thus, is a modifiable risk factor. If this hypothesis is upheld, this will be a significant step forward in MR imaging biomarker qualification in this setting, and will place us strongly to scale-up to larger studies of risk association. The review of literature briefly outlines the existing evidence that weight reduction (surgical or medical) reduces cancer risk. The literature review concludes with a summary of the research directions that emerged and I define my hypothesis.

1.1 OVERVIEW

1.1.1 PANCREATIC CANCER: INCIDENCE, TREATMENT AND SURVIVAL

Pancreatic cancer (PaC) is the 10th most common cause of cancer in the United Kingdom (UK) yet the 5th most common cause of cancer related death. Worldwide there are an estimated 338,000 new cases per year of which 104,000 occur in Europe. Although PaC incidence is fairly stable, there has been a small increase in the UK over the first decade of the 21st century, by 4% in men and 11% in women, to 10.8 and 8.7 new cases per 100,000 population respectively.[2] Overall, in 2011 in the UK there were 8,773 new cases and almost the same number (8,320) of deaths due to PaC. In context, in 2011 in the UK, PaC comprised just 2.7% of new cancer cases but 5.2% of cancer deaths.[3] Across Europe PaC is one of the few types of cancer with an increasing mortality (from 8.1 per 100,000 in 1981 to 9.7 per 100,000 in 2009);[4] PaC is an important cause of cancer related death.

Treatment of PaC depends on disease stage and histological subtype, most commonly pancreatic ductal adenocarcinoma (PDAC). The majority (~80-85%) of patients present with metastatic (~55%) or advanced local disease (~25%) and are not eligible for resection but palliative chemotherapy only.[5, 6] Standard palliative chemotherapy regimens are based on gemcitabine but have limited efficacy although recent advances have shown that the addition of albumin bound paclitaxel to gemcitabine[7] and the chemotherapy regime FOLFIRINOX[8] (a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin) improve survival. Worldwide, standard treatment for patients with resectable disease is surgery followed by adjuvant therapy but geographic variation exists in post-operative adjuvant therapy. In Europe, since the publication of the first study by the European Study Group for Pancreatic Cancer (ESPAC), surgery is followed by post-operative adjuvant chemotherapy.[9] Comparatively, a combination of adjuvant chemoradiotherapy and chemotherapy is favoured in major centres in the United States of America (US).[10-13] Other treatment strategies in this group (such as neoadjuvant chemotherapy) are reserved mainly for trial settings.[14]

Despite advances in systemic therapy and surgical techniques PaC remains the most lethal of common cancers. In the UK, only 18% of patients survive one-year after diagnosis and just 4% survive five-years.[3, 15] Median survival worsens with stage at presentation, survival for locally advanced cancers is 9-15 months and just 3-5 months for metastatic disease.[16] However, for patients with resectable disease, five-year survival is 10-30%[6, 9, 17-19] and median survival between 16 and 24 months. Prognosis is better for early stage disease; 1-year and median survival in lymph node negative and T1 stage disease is 86% and 35 months, and 87% and 33 months respectively;[19] histological margins (R0) are a key

prognosticator of long-term survival with median survival for these patients 20-24 months compared with 8-18 months with resection margin involvement.[19-22] Early stage diagnosis and expedited treatment of PaC is therefore a key strategy in improving outcomes and a key recommendation of the UK All Party Parliamentary Group report into PaC.[23]

1.1.2 A KEY ISSUE IN PANCREATIC CANCER: EARLY DIAGNOSIS

The majority of patients with PaC in the UK present with advanced local or metastatic disease and cannot be offered potentially curative (surgical) treatment. Furthermore, most surgically treated patients typically have advanced tumour stage (~60% are American Joint Committee on Cancer [AJCC] stage T3 or T4), nodal metastases (~70% are node positive) and positive resection margins (~65% are R1).[19, 24] When all new PaC diagnoses are considered, it is clear that the proportion of patients with early stage disease is small and early detection or prevention are key strategies for improving outcomes.

Compared with other common cancers there are significant differences in stage and resectability rates at diagnosis. For colorectal cancer, for example, at diagnosis 75% of patients can be treated surgically, over 30% will be lymph node negative,[25] and at 5 years overall survival is 55%.[26] For breast cancer, over 80% can be treated surgically, almost 40% will be lymph node negative and 5 year overall survival is 85%.[27] A number of factors contribute to poor PaC statistics; early diagnosis is difficult as symptoms often occur only with advanced disease and are non-specific; the anatomical location of the pancreas gland means that small volume local spread swiftly renders the cancer unresectable and that obtaining pancreas tissue for diagnostic purposes is technically difficult. However, fundamentally, it is aggressive tumour biology with exponential growth and early metastases that makes PaC so lethal.

The temporal sequence for the development of PaC indicates that initial mutations take place years before metastatic potential is obtained but circulating (but non-colonising) tumour cells occur early on and the rapid expansion of PaC cells means that most patients will already have undetectable metastases at operation.[28-30] Pancreatic tumours harbour, on average, more than 45 gene mutations of which two are nearly universal.[31, 32] The most frequent and earliest mutation is telomeric shortening and activating mutations in KRAS (~95% of tumours), followed by the inactivation of the tumour suppressor gene INK4A/CDKN2A (90%) in the mid-stage of PaC development, inactivation of TP53 (70%) and SMAD4 (45%) in the late stage are also common. Other lower frequency mutations include BRCA2 (7%), and MAP2K4 (4%).[33, 34] KRAS, CDKN2A, TP53 and SMAD4 are founder mutations that occur in PaC precursor lesions known as pancreatic intraepithelial neoplasia (PanINs). Intriguingly, there is evidence that these are initially slow growing neoplasia[35] and genomic evaluation estimates that the founder mutations take place a

decade before metastatic potential is obtained.[28]   Therefore, there may be a window of opportunity for early identification and treatment with the hope of improved outcomes. However, in clinical practice, identification of premalignant lesions, except in cystic neoplasia,[36] is not yet possible.

Clinical experience and animal models demonstrate that metastases in PaC often occur prior to a radiologically visible mass. [30] As KRAS mutant PanIN cells in circulation may be incapable of colonising distant sites, the critical issue becomes the timing of clinically relevant circulating tumour cells. Mathematical modelling of tumour growth and metastases by Haeno et al has demonstrated that the rate of cell division in PaC is exponential and most patients will have metastases at presentation.[29] Although dismal, these experimental findings echo the experience of oncologists and surgeons treating this disease. The findings reinforce the need for effective systemic therapies, early detection, prevention and perhaps suggest that trials that aim to improve resectability by local down-staging may have only a marginal effect on overall mortality. However, due to the low overall incidence (14 per 100,000), large-scale screening programmes using computerised tomography (CT), Endoscopic Ultrasound (EUS) or magnetic resonance imaging scans are not feasible,[37] a key strategy in improving PaC outcomes is therefore characterisation and identification of the high-risk population.

1.1.3 HIGH-RISK GROUPS FOR PANCREATIC CANCER DEVELOPMENT

As a strategy to reduce mortality, there are two reasons to identify a population at high-risk of PaC. Firstly, the potential for early identification and therefore improved outcome post diagnosis, and secondly, to reduce PaC incidence by implementing strategies to modify risk. Yet, there are currently only a handful of indications for PaC screening.[38]

Putative risk factors for the development of PaC include environmental, lifestyle, and genetic factors. The germline mutations in Peutz-Jeghers syndrome, BRCA mutations, familial atypical mole melanoma (FAMMM), lynch syndrome (hereditary non-polyposis colorectal cancer, hereditary pancreatitis and cystic fibrosis are all linked with an increased risk of pancreatic cancer. In particular, patients with Peutz-Jegher syndrome have a PaC risk, 76 times that of the general population.[39] Additionally, there is a cohort of patients with a strong family history of PaC without identified genetic abnormalities. In the Pancreatic Cancer Cohort Consortium study a moderate risk increase (OR, 1.7, 1.19-2.91) was associated with a family history of PaC.[40] A prospective registry-based study identified a risk increase of 4.6-fold for 1, 6.4-fold for 2 and 32-fold for 3 affected first degree relatives.[41] Novel genomic risk factors have also been identified through advances in genome analysis techniques. Petersen et al in a genome wide association study (GWAS) of 3,851 cases and

3,934 controls yielded three new genomic regions associated with the risk of PaC 13q22, 1q32 and 5p15.[42]

A number of environmental risk-factors have been proposed, accepted ones include smoking, occupational exposure and alcohol intake.[35] Smoking remains the most important risk factor with 4.3-fold increase in risk[43] although the temporal relationship between quitting and risk returning to normal is unclear, calculations range from 5-years to 20-years.[44, 45]

Chronic pancreatitis and diabetes are associated with an increased risk of PaC although these associations are complex. Patients with chronic pancreatitis have a 5% risk over 20-years of developing PaC and a 13-fold relative risk increase,[46] but this relationship is confounded by the presence on the causal pathway for both diseases of common risk factors (alcohol and smoking). Diabetes is both an aetiological factor in the development of PaC and an early manifestation of PaC. The most recent meta-analysis of the relationship between diabetes and PaC found a modest risk increase (RR 1.97, 1.78-2.18).[47] However, diabetes is also an early manifestation of PaC as new onset diabetes is present in 40% of diagnoses.[48, 49]

Overall, the difficulty in identifying the at-risk individual was highlighted by Klein and colleagues. In a recent analysis of the pooled PanScan study these authors combined non-genetic and genetic risk factors for pancreatic cancer and derived absolute risk based on population incidence rates. In a United States (US) population they estimated that less than 3/1,000 had a greater than 5% predicted lifetime absolute risk.[50] Therefore, although screening of high-risk groups is appealing as five-year survival is better for early-stage disease, this is not yet feasible. Additional considerations are that early detection of PaC is difficult and surgical treatment associated with significant complications. Confirming early-stage PaC presents a diagnostic challenge as the anatomical position of the pancreas means tissue is difficult to obtain, there is no reliable diagnostic biomarker and imaging can be equivocal. When PaC is suspected but the diagnosis uncertain, the high mortality and morbidity associated with surgical treatment must be weighed against the benefit of early treatment. As a parallel, the risks of overtreatment in breast cancer are smaller than for pancreatic resections, yet the overall benefit of screening for this cancer in the UK are still debated.[51]

Alternatively, the identification of high-risk groups is an opportunity to implement risk-reducing strategies. Many risk factors for PaC (genetic, familial) are not currently amenable to this approach. However, environmental and lifestyle factors such as smoking, chemical exposure and obesity may be. As a risk factor for PaC the increased risk associated with obesity is small (RR 1.19, 95% CI 1.09-1.31)[52] and the overall high incidence of obesity currently precludes targeted intervention. Yet, we know that for a given BMI there is considerable heterogeneity in the distribution of adipose tissue. For cardiovascular and

metabolic disease, this variation in adipose tissue distribution, in part, explains the differences in disease risk for a given BMI.[53-55] There is therefore an opportunity to extend this hypothesis to PaC risk, to better define the risk attributable to excess adiposity with the potential to identify a modifiable target for risk-reducing intervention.

1.1.4 EXCESS ADIPOSITY AND PANCREATIC CANCER

A 2007 report from the World Cancer Research Fund (WCRF) reviewed 23 cohorts and 15 case-control studies and concluded that there was evidence of an increased risk of PaC with increased body fatness.[56] Simultaneously, Renehan et al reported on the relationship between BMI and a number of cancers using a standardised approach and found a modest relationship between BMI and PaC in women but not men.[57] In the intervening period, updated evidence, a further meta-analysis and 3 pooled-analyses, has extended the association to include men.[58-61] This updated evidence is now considered by the WCRF to be 'convincing' of a link between excess body weight and PaC.[62]

The most common surrogate anthropometric measure of obesity used in the epidemiological literature is BMI. However, BMI does not distinguish lean body mass from adipose tissue and does not reflect adipose tissue distribution.[63] Adipose tissue can be subdivided into visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Measures of waist circumference (WC) and waist-to-hip (WHR) ratio have been proposed as better representing abdominal adipose tissue distribution (VAT). In turn VAT may be a better predictor of excess adiposity related complications such as insulin resistance.[64, 65] A smaller volume of literature reports associations between these measures and PaC incidence. Three individual studies have found a significant association for WC and/or WHR and PaC incidence but not for BMI.[66-68] One cohort study[69] and one pooled-analysis[58] have found WC and WHR respectively to be a risk factor for PaC independent of BMI.

Table 1.1 summarises the four meta-analyses and four pooled-analyses investigating the association between BMI and PaC incidence.[57-61, 70-72] Risk appears to be similar for both men and women, although findings are inconsistent.[59, 70] Diabetes partly attenuates but does not remove risk in adjusted models and is itself a risk factor.[73] The most recent and largest (by number of cases) meta-analysis by Aune et al demonstrated a non-linear association with a steep rise in risk for BMI > 35 kg/m^2.[59] When adjusted for smoking, risk associated with a 5 kg/m^2 was limited to non-smokers (RR 1.11, 95% CI 1.04-1.17) a finding supported by the 3 pooled-analyses.[58-61]

One meta-analysis and two pooled-analyses have investigated the association between WC and PaC incidence. Aune et al performed a meta-analysis of 5 cohort studies and 949 cases of PaC and found a RR of 1.11 (95% CI, 1.05-1.18) per 10 cm increment in WC. This effect was statistically significant in women (RR 1.14, 95% CI 1.02-1.28) but not

men (RR 1.13, 95% CI 0.89-1.44).[59] Two separate pooled-analysis reported positive associations when comparing highest verses lowest categories of WC, but these were not statistically significant.[58, 60]

Results of the one meta-analysis and two pooled-analyses investigating the association between WHR and PaC incidence are similar to those for WC. Aune et al analysed 4 cohort studies and 1047 cases of PaC in a meta-analysis and found a RR of 1.19 (95% CI, 1.09-1.31) per 0.1 unit increment in WHR. Risk was similar for men (RR 1.20, 95% CI 0.96-1.50) and women (RR 1.17, 95% CI, 1.00-1.36) but only significant in the latter, possibly due to only 1 study reporting on men.[59] Two separate pooled-analyses reported significant associations when comparing highest verses lowest categories of WHR that remained after adjustment for BMI and diabetes. Genkinger et al found a RR of 1.35 (95% CI, 1.03-1.78) and additionally in a model adjusted for BMI risk remained (RR 1.34, 95% CI 1.00-1.79).[58] Arslan et al found a RR of 1.71 (95% CI, 1.27-2.30), this remained after adjustment for diabetes.[60]

An important question for clinicians and researchers investigating the link between excess adiposity and PaC is exposure duration and risk increase. Three epidemiological studies implicate early adulthood obesity in PaC risk[52, 58, 74] although results are inconsistent.[75-77] Li et al suggested that weight gain in earlier adulthood was associated with increased risk for excess adiposity related pancreatic cancer.[74] Similarly, in the pooled analysis of 14 cohort studies by Genkinger et al, 11 studies provided data on weight at age 18 or 21 years (termed early adulthood weight). The authors found that BMI in early adulthood was positively associated with PaC risk, and when adjusted for BMI at baseline, this risk remained. Risk was particularly high in this cohort for patients who were overweight in early adulthood and obese at baseline (RR 1.54, 95% CI 1.24-01.93).[58] Stolzenberg-Solomon investigated the incremental change in PaC risk with duration of BMI>25kg/m^2; an increased risk (Hazard ratio 1.6, 95%CI 1.02-1.09) associated with each 10-year increment in duration of being overweight or obese.[52]

The proportion of the population within the UK that are overweight or obese continues to rise.[78] In 2008, 66% of men and 57% of women had a BMI greater than 25 kg/m^2 [79] and the United Kingdom now has the second highest number of new cancer cases attributable to excess BMI in Europe.[80] However, as the overall incidence of PaC is only 10 per 100,000 population and only 12% of pancreatic cancers are estimated to be attributable to excess body weight,[81] BMI is not a refined enough marker of PaC risk. Therefore, we need to identify biomarkers on the causal pathway between anthropometric measurements of excess adiposity and PaC that better identify the high-risk group.

Table 1.1: Relative risk and 95% CI of pancreatic cancer per unit increase in BMI (5 kg/m^2)

First author (year)	Number of studies	Number of cases	Risk ratio by gender (95% CI)	Combined Risk ratio (95% CI)	Results from studies adjusted for smoking	Results from studies adjusted for diabetes
Meta-analyses						
Aune (2012)[59]	24	9,504	Men 1.13 (1.04-1.22) Women 1.10 (1.04-1.16)	1.10 (1.07-1.14)	Yes 1.14 (0.96-1.17) No 1.09 (1.04-1.14)	Yes 1.12 (1.05-1.20) No 1.11 (1.07-1.14)
Renehan (2008)[57]	16	4,443	Men 1.07 (0.93-1.23) Women 1.12 (1.03-1.23)	Not given	Separate analysis for studies adjusted/ not adjusted for smoking not performed	Separate analysis for studies adjusted/ not adjusted for diabetes not performed
Larsson (2007)[70]	21	8,062	Men 1.16 (1.06-1.17) Women 1.10 (1.02-1.19)	1.12 (1.06-1.17)	1.12 (1.06-1.17) All studies adjusted for smoking	Yes 1.15 (1.08-1.23) No 1.06 (1.0-1.13)
Berrington de Gonzalez[a] (2003)[71]	14s	6,391	Men 1.03 (1.01-1.06) Women 1.02 (1.00-1.03)	1.02 (1.01-1.03)	Yes 1.03 (1.02-1.03) No 1.00 (0.96- 1.03)	Yes 1.03 (1.00-1.06) No 1.02 (1.02-1.03)
Pooled-analyses						
Genkinger (2011)[58]	14	2,136	Men 1.14 (1.01-1.29) Women 1.13 (1.01-1.21)	1.14 (1.07-1.21)	Never 1.19 (1.08-1.31) Former 1.22 (1.10-1.34) Current 1.07 (0.95-1.21)	Results were similar when adjusting for diabetes, but results are not shown
Jiao (2010)[61]	7	2,454	Men 1.06 (0.99-1.13) Women 1.12 (1.05-1.19)	1.08 (1.03-1.14)	Never 1.15 (1.06-1.25) Former 1.11 (1.03-1.20) Current 1.00 (0.91-1.11)	Results were similar when adjusting for diabetes, but results are not shown
Arslan[b] (2010)[60]	13	2,170	Men 1.33 (1.04-1.69) Women 1.34 (1.05-1.70)	1.33 (1.12-1.58)	Smokers 1.14 (0.91-1.78) Non-smokers 1.37 (1.06-1.78)	Model adjusted for diabetes 1.21 (1.01-1.44
Parr (2010)[72]	39	301	Men 0.85 (0.63-1.16) Women 1.17 (0.91-1.51)	1.02 (0.83-1.25)	Model adjusted for smoking status	Not adjusted for diabetes

[a] Berrington de Gonzalez et al considered unit increase in BMI to be1 kg/m^2
[b] Arslan et al risk ratios of highest vs lowest BMI quartile category

1.1.5 ADIPOSE TISSUE DISTRIBUTION

Body Mass Index as an approximation of adiposity does not distinguish between lean body mass and adipose tissue, or between VAT and SAT. Measures of abdominal adipose adiposity, WC and WHR, comprise both SAT and VAT and the relative proportion of these adipose tissue depots vary significantly between individuals. Adipose tissue can be further divided by location into non-ectopic (SAT) and ectopic fat. Ectopic fat (including VAT) surrounds, and is stored within, organs and blood vessels. This variation is important as evidence from the past ten years has demonstrated that adipose tissue has important endocrinological, immunological and inflammatory functions that vary between different adipose tissue depots.[82]

Variation in adipose tissue distribution is important in the risk of developing cardiovascular and metabolic disease.[83] A subgroup analysis of the Framingham Heart Study explored the relationship between VAT, SAT and metabolic risk factors. VAT was associated with more adverse levels of metabolic risk factors compared with SAT. The study included 3001 participants who had undergone CT analysis of VAT and SAT volumes. Both SAT and VAT correlated with metabolic risk factors, but correlations with VAT were significantly stronger than those for SAT. Stratification by VAT consistently explained more of the variation in the presence of metabolic risk factors than other anthropometrics (BMI and WC).[53] Comparatively, SAT was no better at explaining this variation than simple anthropometrics. Further analysis by the same group has extended these findings to show that the correlation with insulin resistance (measured by homeostasis model assessment of insulin resistance [$HOMA_{ir}$]) is stronger for VAT than SAT.[84]

As methods of quantifying adipose tissue deposition have developed, the importance of local deposition of adipose tissue, a toxic paracrine effect, has emerged. Pericardial fat is hypothesised to increase coronary artery disease and is associated with increased arterial calcification and cardiovascular disease in models adjusted for BMI and abdominal waist circumference.[85] Perirenal fat is hypothesised to affect renal function due to a local compressive effect on renal veins, and was found to be associated with high blood pressure and chronic kidney disease independent of BMI and VAT.[86]

Excess adipose tissue is therefore a heterogeneous condition in which individuals with similar levels of BMI may have distinct metabolic and cardiovascular disease risk dependant in part on variation in adipose tissue distribution. This hypothesis is now being extrapolated to cancer risk with the effects of locally deposited fat implicated in a potentially tumour promoting environment.

1.1.6 ECTOPIC ADIPOSE TISSUE AND LOCAL INFLAMMATION

The accumulation of adipose tissue in organs occurs in conjunction with the infiltration of immune cells. Although adipose tissue is mainly comprised of adipocytes, other cell types are required for growth and function. In obese individuals these cell types change to pro-inflammatory immune cells and there is upregulation of pro-inflammatory cytokines. The result is a pro-inflammatory local environment known as the 'inflammasome'.[87]

In obese individuals adipose tissue is infiltrated by large numbers of macrophages, more abundant in visceral than subcutaneous fat. The macrophage subset changes in association with increased adipose tissue. In lean mice, macrophages express genes associated with an M2 phenotype.[88] M2 macrophages upregulate the production of the anti-inflammatory cytokines Il-10 and downregulate pro-inflammatory synthesis.[89] In obese mice, macrophages express genes associated with an M1 phenotype.[88] These produce pro-inflammatory cytokines (including IL-6 and TNF), express inducible nitric oxide synthase and produce reactive oxygen species.[89] There are concomitant changes in the T-cell population with obesity that contribute to inflammation. The adipose tissue of lean mice contains a greater proportion of CD4+ regulatory T cells than obese mice where CD8+ effector T cells predominate. CD8+ effector T cells can initiate recruitment and activation of macrophages and a pro-inflmmatory cascade.[90, 91] The pro-inflammatory state not only acts locally but may contribute to systemic inflammation associated with insulin resistance.[92]

Inflammation is putative mechanism in the development of excess adiposity-related cancers.[93, 94] The importance of local fat, in the context of PaC, is under-researched but established in other cancers and obesity-driven metabolic diseases. An example of the disease model of excess adiposity, local inflammation and cancer, is the development of hepatocellular carcinoma (HCC) secondary to non-alcoholic fatty liver disease (NAFLD). In states of excess energy, fatty acids are stored as triglycerides within hepatocytes and around hepatocytes in adipocytes.[95] This leads to the production of pro-inflammatory cytokines and recruitment of immune cells. In a proportion of the population this progresses into non-alcoholic steatohepatitis (NASH), a major risk factor for HCC.[96] As the pancreas is considered to also be an ectopic fat store, and as there are established associations between obesity and PaC, here, we extend the obesity-local inflammation- cancer hypothesis to the pancreas.

1.1.7 INTRAPANCREATIC FAT

Schaefer was the first to note a relationship between increased body weight and increased pancreatic weight at post-mortem in 1926.[97] Ogilvie et al extended this to link pancreatic fat and obesity they described 9% fat in 'lean' cadavers compared with 17% fat in 'obese'

cadavers.[98] Fat infiltration in the pancreas is usually a diffuse process occurring uniformly throughout the gland. It is seen on histopathological analysis in the form of adipocytes within (intralobar) and between (interlobar) lobules, although focal fatty infiltration has also been documented.[99] The importance of intra-pancreatic fat is comparatively under-researched, than for example hepatic fat, due in part to the morbidity associated with obtaining pancreatic tissue in humans.

The associations of intra-pancreatic fat with other markers of excess adiposity are inconsistent due to the heterogeneity of inclusion criteria, methods of quantification and small study groups. Nomenclature is also variable but the degree of pancreatic fat is typically described as a fraction of the whole gland and termed pancreatic fat fraction (PFF). Most studies have found an association between BMI with PFF. Lingvey et al found a 7-fold increase in PFF in patients with a BMI 32.4 ± 6.1 kg/m^2 compared with BMI of 22.2 ± 1.6 kg/m^2.[100] Similarly, Maggio et al found 4.8 ± 1.9% and 3.6 ± 0.9% PFF in obese (BMI 30.3 ± 5.4 kg/m^2) and lean (18.9 ± 1.9 kg/m^2) adolescents respectively.[101] In contrast, Patel et al in a cohort of patients with non-alcoholic fatty liver disease and Lee et al in 293 overweight or obese patients found no association between BMI and PFF.[102, 103]

An association between increased VAT and PFF has been reported by some,[103-105] but not all.[106, 107] Rossi et al found VAT to be the main predictor of PFF in a study assessing body fat distribution, inflammatory markers, adipocytokines in obese men and women.[105] Heni et al found a significant correlation between VAT and PFF in a model adjusted for age and gender.[104] Lee et al assessed pancreatic fat using USS in a multivariable model and found VAT to be the determinant factor in PFF over BMI.[103] Conversely, both Van der Zijl in a study of age and BMI matched individuals and Hannukainen in a study of monozygotic twins found no associated between VAT and PFF as measured by MRS.[106, 107]

Further research into the association of PFF with obesity distribution is therefore warranted. One theory of ectopic fat deposition is that excess free fatty acids are first stored subcutaneously, once this volume is exhausted, adipose tissue then accumulates as ectopic fat within organs and as VAT. This is consistent with studies finding a closer correlation between PFF and VAT than PFF and SAT[104, 105, 108] and may have implications for identifying the population at high risk of intra-organ fat deposition for further assessment.

1.1.8 PANCREATIC STEATOSIS AND DIABETES

Research into the effect of pancreatic steatosis on decreasing pancreatic β cell function, termed by some as 'lipotoxicity',[109] has inconsistently demonstrated an association but not causative link. Heni et al and Van der Zijl et al found increased PFF to be negatively associated with insulin secretion in subjects with impaired glucose tolerance (IGT) and impaired glucose fasting (IGF).[104, 106] Similarly, Wu et al and Ou et al found an association

between impaired glucose metabolism in patients with increased pancreatic fat diagnosed on USS.[110, 111] Tusheizen et al reported increased PFF in age and BMI matched type II diabetics compared with controls concluding that PFF may have a direct effect on pancreatic function.[112] Conversely, Saisho et al found no correlation between CT derived pancreatic fat and type-2 diabetes.[113, 114] Taken together, the inconsistent study findings reflect the heterogeneity in design and outcomes. This pattern of findings is consistent with much of the metabolic literature that associates excess adiposity with impaired glucose tolerance and subsequently type II diabetes; there remains debate over whether intraorgan fat within the liver and muscles contributes to insulin resistance or is just a marker of other pathological processes.

1.1.9 INFLAMMATION AND ADIPOSITY IN PANCREATIC CANCER DEVELOPMENT

Chronic pancreatitis, a progressive inflammatory disorder characterised by disregulated secretion and premature activation of pancreatic enzymes, is an established risk factor for the development of PaC. Meta-analysis by Raimondi et al. of 6 cohort studies and 1 case-control study found a pooled RR of 13.3 (95% CI, 6.1-28.9) for the risk of PaC with a history of chronic pancreatitis.[46] Although the exact mechanisms by which chronic inflammation leads to the development of PaC are not clear, it is generally accepted that inflammation results in cellular insult and the progressive accumulation of genetic defects. This manifests as the PaC precursors, PanINs, which progress through stages of cytological and architectural changes associated with different genetic mutations.[115]

PaC had historically been thought to originate in pancreatic ductal cells, this paradigm is now being challenged to provide a link with chronic pancreatitis (a predominantly acinar cell disease). In a pathway analogous to a number of other chronic inflammation related cancer precursors (e.g. Barretts oesophagus) acinar to ductal metaplasia has been observed in rodent models of pancreatitis. PaC has been observed to originate from acinar cells through this transition as a result of both genetic changes (activation of oncogenic KRAS and loss of tumour suppressor barriers) and inflammation typical of pancreatitis.[116]

The link between excess adiposity and chronic pancreatitis is less clear. Excess abdominal adiposity, although not total adiposity or BMI, is linked to the risk of acute pancreatitis after controlling for confounding factors.[117] This holds true for both gallstone and non-gallstone pancreatitis. Additionally, obesity increases the severity of acute pancreatitis, possibly due to an increased systemic inflammatory response.[118] However, epidemiological evidence does not support a link between excess adiposity and chronic pancreatitis.[46]

Inflammatory pathways are considered key in the development of PaC and are recapitulated in animal models of PaC development. Oxidative stress and the generation of

reactive oxygen species (ROS) and reactive nitrogen species are key in the pathophysiology of acute and chronic pancreatitis and perpetuate acinar cell necrosis and fibrosis.[119] Inactivation of TP53INP1, a protein that controls oxidative stress, accelerates pancreatic cancer development in a KRAS mutant background.[120] COX-2 is activated by inflammatory cytokines and its expression is upregulated in both pancreatitis and pancreatic cancer.[121] Models of PaC development, using genetically engineered mice, manipulate these inflammatory pathways to induce PaC (summarised by Pinho et al.[116]), other models have used direct carcinogenic injections that mimic pancreatitis to cause pancreatic cancer development.

1.1.10 INFLAMMATION, OBESITY AND PaC DEVELOPMENT IN ANIMAL MODELS

Animal models of pancreatic cancer development offer the opportunity to study the effect of excess adiposity on PaC. These models have demonstrated that excess adiposity is associated with accelerated progression of precancerous and cancerous neoplasia and inflammatory changes. **Table 1.2** summarises these studies and results.

Lashinger and colleagues investigated the effect of calorie restriction treatments in a COX-2-driven pancreatitis to PaC model.[122] Compared to an isoenergetic control diet, calorie restriction was associated with decreased serum IFG-1, less pancreatic ductal lesion formation and lower grade dysplastic severity. To demonstrate that the tumours produced by the COX-2-driven pancreatitis model were IGF-1 sensitive, after injection of these tumours into IGF-1 deficient mice and controls, tumour burden was significantly less in the IGF-1 deficient mice.[122] Further research by the same group found that rapamycin, a drug that mimics the effects of calorie restriction by suppressing mammalian target of rapamycin (mTOR), reduced pancreatic tumour volume in a PaC transplant murine model.[123] Dawson et al in a conditional kras[G12D]/PDX-1-Cre mouse model (mutant) with a normal mouse (wildtype) control arm investigated the effect of a high fat, high calorie (HFHC) diet on chronic pancreatitis and the development of PaC precursors, PanINs.[124] Mice fed the HFHC diet gained weight and this was associated with metabolic disturbances. Pancreatic tissue from the mutant and wildtype HFHC diet fed mice exhibited features consistent with the chronic pancreatits; inflammatory cell infiltration, stromal fibrosis, acinar cell loss. However, significant increases in cytokine infiltration were seen only in the mutant HFHC fed mice. These results are consistent with our hypothesis of intra-organ inflammation secondary to obesity although the authors do not comment on the presence of adipocytes within pancreas specimens. The presence of inflammatory cells in the wildtype mice fed a HFHC diet indicates that this process can occur without the genetic changes that characterise PaC and PanINs. Mutant mice fed a HFHC diet exhibited a greater number of, and more advanced PanINs than the mutant mice fed the control diet. The increased frequency of more

advanced PanINs in the mutant mice indicate this inflammation may have a role in the progression of premalignant neoplasia of the pancreas.

Using the same genetic model, Lanza-Jacoby and colleagues investigated the effect of calorie restriction on the development and progression of PanINs. Mice fed a control diet gained weight and had increased numbers of more advanced PanINs than mice fed either of two diets. Additionally, mice on daily energy restriction diet had a significant reduction in serum IGF-1 levels.[125]

The kras[G12D]/PDX-1-Cre mouse model is however limited in that it develops only premalignant neoplasms. The kras[G12D]/PDX-1-Cre/Ink4a/Arf[lox/+] mouse model develops PanINs which progress to PaC. Lanshinger et al. tested the effect of a calorie restricted diet, diet induced obesity (DIO) verses a control diet in this mouse model. In mice slaughtered at 10 weeks pancreata of DIO obesity had the highest degree of fibrosis and high-grade inflammatory cell infiltration and these mice more frequently developed PaC.[126]

Two authors have investigated the effect of diet on chemically induced PaC. In a rat model of 7,12-dimethylbenzanthracene (DMBA)-induced PaC Z'grannen and colleagues found a high fat and high protein diet increased the prevalence of cancer and dysplasia at 9 months compared to controls.[127] Hori et al. demonstrated in a hamster model that the combination of a high-fat diet and N-nitrosobis-(2-oxopropyl)amine, induced PaC. The control arm, fed a normal diet did not develop PaC. In addition, a high-fat diet was associated with adipocyte infiltration of the pancreas and increased pancreatic cell mRNA expression of the inflammatory related genes monocyte chemoattractant protein 1, IL-1β and COX-2. Expression of mRNA in the pancreatic cells was greater for the leptin, plasminogen activator inhibitor 1, and fatty acid synthesase.[128]

The presence of inflammatory infiltrate is not a benign process within the pancreas. Pancreatic acinar cells with the kras[G12D] mutation cross-talk with immune cells, causing local inflammation and this promotes acinar-to-ductal metaplasia and PanIN development. The kras[G12D] mutation induces expression of intercellular adhesion molecule-1 (ICAM-1) and this promotes the infiltration of macrophages. This is associated with greater pancreatic stromal remodelling possibly due to increased production of cytokines such as TNF-α and proteases including matrix metalloproteinase 9 (MMP9). Importantly, depletion of macrophages in this model lead to a reduced stromal remodelling and delayed acinar-to-ductal cell metaplasia.[129] In a model without PaC driver mutations, rats fed a HFHC diet accumulate triglycerides within acinar cells with subsequent pancreatic fibrosis and acinar cell injury.[130]

Taken together, this body of research provides evidence that calorie intake influences the development and progression of PaC in mice models. However, although recent models more accurately replicate the same genomic mutations seen in human PaC, there are fundamental differences that limit their applicability and emphasise for human

validation. Firstly, the time course for development of PaC in these models (often within 20 weeks) does not mirror the gradual decade long accumulation of genetic mutations seen in PaC.[28] Secondly, current epidemiological evidence suggests that obesity-related pancreatic cancer risk is greatest with early adulthood obesity, the human model therefore has a length of exposure to excess adiposity of at least 10 years in comparison with the short exposure seen in animal models. Finally, by mimicking the genomic variations seen in established PaC to induce PaC these models study only later stages of PaC development in humans and not the initiating genomic mutations.

Table 1.2: Animal models investigating the influence of diet, weight change and associated pathways on pancreatic neoplasm development.

First Author (year)	Model	Phenotype	Intervention	Outcome
Dawson (2013)[124]	LSL-Kras^{G12D}/Pdx-1-Cre mice	PanIN with low penetrance of PaC (5% at 1 year)	High fat, high calorie (HFHC) diet vs control diet in both mutant and WT mice	Mutant and WT HFHC mice gained weight. Pancreatic inflammation increased in HFHC mice. Pancreatic cytokines increased in mutant HFHC mice but not WT mice. Increased pancreatic stellate cell activation in HFHC mice. Acceleration of PanIN development in mutant mice on HFHC diet
Lanza-Jacoby (2013)[125]	LSL-Kras^{G12D}/Pdx-1-Cre mice	PanIN with low penetrance of PaC (5% at 1 year)	CD vs IER vs CCR	Decreased frequency of PanINs in IER and CCR groups. Decreased IGF-1 in CCR group. CCR decreased mTOR phosphorylation
Lashinger (2011)[122]	(a) BK5.COX-2 (b) Orthotopic transplant of dysplastic cells into loxP+/+ Cre+/− (LC) mice and loxP+/+ Cre−/− (LID) (IGF-1 deficient) mice	(a) Chronic pancreatitis and ductal dysplasia (b) Anaplastic tumours	(a) CD vs CR diet (b) LID and LC mice fed control diet	(a) Decreased pancreatic ductal lesion formation and dysplastic severity in CR mice (b) Tumour burden significantly less in LID (IGF-1 deficient) mice
Lashinger (2011)[123]	Orthotopic model in male C57BL/6 mice	Orthotopic PaC	(a) Weeks 1-20 CR vs CD (b) Rapamycin administration to half of CD group and all of CR group	(a) Improved glucose tolerance and lower IGF-1 in CR group (b) Tumours in rapamycin treated mice and CR treated mice grew slower than CD mice. CR restriction associated with lower adipocyte infiltration than CD.
Lashinger (2013)[126]	(a) LSL-Kras^{G12D}/Pdx-1-Cre/Ink4a/Arf$^{lox/+}$ (b) Orthotopic tumour transplant into IGF-1 deficient (LID) and WT mice	(a) PanIN and PaC (b) Orthotopic PaC	(a) CR vs CD vs DIO (b) LID mice received either IGF-1 infusion or placebo	(a) Increased frequency of PaC and AKT/mTOR signalling in DIO group (b) Tumour weight lower in LID and placebo mice than LID and IGF-1 or WT mice
Matsuda (2014)[130]	Diabetic (Lepfra) Zucker rats		CD vs HFHC diet	HFHC fed rats accumulated lipid droplets in acinar cells and developed fibrosis and chronic pancreatitis stigmata
Z'grannen (2001)[127]	DMBA injection in pancreas of rats	Dysplastic lesions and PaC	CD vs HFHC diet	HFHC diet fed mice gained weight. Greater prevalence of PaC in HFHC rats.
White (2010)[131]	Orthotopic model in female C57BL/6 mice	Orthotopic PaC	CD vs DIO	Larger tumours in overweight mice; tumour size correlated with body-weight
White (2012)[132]	Orthotopic model in male C57BL/6 mice	Orthotopic PaC	High-fat vs low-fat diet	Body-weight but not diet was correlated with tumour-weight

Control Diet (CD), Calorie Restriction (CR), High fat high calorie (HFHC), Intermittent Energy Restriction (IER), Continuous Calorie Restriction (CCR), Wildtype (WT), Diet Induced Obesity (DIO)

1.1.11 GENETIC SUSCEPTIBILITY TO PaC

Genes associated with inflammation, excess adiposity and diabetes are now identified by genomic analyses to be associated with an increased risk of developing PaC.

Tang and colleagues aimed to examine genetic pathways that modified the associations of obesity and diabetes with PaC.[133] The study included 2,028 cases and 2,109 controls and examined 197 pathways with 10 to 500 genes each. They found an interaction of the chemokine signalling pathway with obesity in modifying PaC risk. The identified genes suggested a central role of the NF-κB pathway an important pathway in the activation of cyclo-oxygenase and Nitric Oxide, important mediators of chronic inflammation.[134, 135] A further finding was of an interaction between a calcium signalling pathway and diabetes and PaC risk. Interestingly, the significant gene is this pathway was GNAS. This gene is found with a high frequency of mutations in IPMNs.[136] Studies in mice indicate that mutations in this gene lead to obesity, glucose intolerance and insulin resistance.[137] The authors concluded that variants in GNAS may contribute to diabetes associated PaC risk.

A case-control candidate gene association study by Reid-Lonabardo et al. used 1352 pancreatic cases and 1189 controls from a single centre and examined 102 gene codes for proinflammatory mediators, inhibitors, or activators of NF-κB.[138] They identified four single nucleotide polymorphisms (SNPs) on the NO51 gene and one on the CD101 gene that correlated with the risk of developing PaC. However, the study failed to replicate the findings using data from the PanScan PaC population.[138]

The fat mass and obesity-associated (FTO) gene has been identified as influencing excess adiposity and diabetes risk. Its component SNP variants are associated with differences in various excess adiposity associated traits including hip circumference and energy intake. In the context of PaC risk, these SNPs have been the interest of a number of case controlled studies. Two of these studies have investigated FTO rs9939609. Lin et al. found a significant association with the FTO rs9939609 A allele variant and PaC risk in a model adjusted for smoking, BMI, and age in a Japanese cohort.[139] These findings were consistent with Tang et al. in a study of Caucasians although the influence of this gene was limited to cases with a BMI of over 25 only.[140] The differences in the two studies may be due to differences in BMI acquisition. Epidemiological studies have previously shown that there is confounding when BMI at the time of diagnosis is used due to the weight loss commonly associated with PaC.

Pierce et al. examined associations between 37 genetic variants, known to increase susceptibility for type-2 diabetes, and PaC risk in a case control study of 1,763 cases and 1,802 controls. Three SNPs were association with an increased risk of PaC. Another variant of the FTO gene, the FTO allele SNP rs8050136, the MTNR1B allele SNP rs1387153 and

the glucose-raising allele of MADD SNP rs11039149. One SNP, BCL11A rs243021 was inversely associated with PaC risk.[141] Comparatively, Prizment et al. examined associations between 10 genetic variants, associated with increased risk of type-2 diabetes, and PaC risk in a case control study of 162 cases and 540 controls. The GCKR allele SNP 780094 was associated with increased PaC risk. The FTO allele SNP rs8050136 was not associated with increased risk.[142]

The studies identified here use different methods to identify genomic variants associated with increased PaC risk and this may explain the different results. While GWAS may identify previously unidentified variants, the required rigorous statistical corrections for multiple testing means that some genetic associations are likely to be missed.[42] Comparatively, more refined studies may fail to adequately assess the large number of genetic variants that are likely to make up risk. For example, studies discussed here have assessed the possible influence of different FTO variants, a candidate gene that interlinks excess adiposity and type-2 diabetes. Overall, these studies support the epidemiological evidence of links between type II diabetes, obesity and the development of PaC. The interaction of these genomic variants and environmental factors may lead to further refinement of the at risk population.

1.1.12 PANCREATIC FAT AND HUMAN PANCREATIC NEOPLASMS

Two papers link intra-pancreatic fat deposition, above other adipose tissue depots, with neoplasia development and progression in the pancreas. Rebours et al classified intra-lobular and inter-lobular pancreatic fat (no adipocytes, scattered adipocytes, numerous adipocytes) and fibrosis in histological specimens of patients undergoing surgery for pancreatic neuroendocrine tumours (pNETs), measured BMI and quantified visceral and subcutaneous adipose tissue from pre-operative CT images. The authors related these markers of excess adiposity to the presence and grade of the pre-malignant PaC precursor, PanINs. Multivariate linear regression identified intra-lobular pancreatic fat and intra-pancreatic fibrosis as factors associated with number and severity of PanINs.[143] A similar case-matched study by Hori et al compared intra-pancreatic fat deposition in resected pancreatic ductal adenocarcinoma specimens with pancreatic resections for other cancers. The authors found a greater intra-pancreatic fat deposition in the pancreatic ductal adenocarcinoma specimens that in controls. This association remained after adjustment for other obesity related confounders (BMI, diabetes mellitus).[144]

Although these studies provide the first in-human evidence that intra-pancreatic fat may better quantify the risk attributable to excess adiposity of developing PaC, there are important limitations. Firstly, both studies performed a histological assessment of pancreatic specimens from patients with known pancreatic disease this methodology introduces the

issue of reverse causality, that intra-pancreatic fat deposition is secondary to disease. There may be some evidence of this in the study by Rebours et al, where pancreatic fibrosis was also independently associated with PanIN presence and severity. Secondly, the study by Rebours et al associated only PanINs with intra-pancreatic fat deposition.[143] Although PanINs are considered the precursor to PaC the speed and frequency of development from PanIN precursor to invasive cancer is unknown. An analogous cancer precursor in humans may be colorectal adenomas. The life-time risk of this colorectal cancer precursor is 40% for the Western population, but only 3% progress to invasive cancer.[145, 146]

Despite the limitations outlined above, these studies provide human evidence of an association between intra-pancreatic fat and the progression and development of PaC, supporting our overall hypothesis. However, it remains to be demonstrated that intra-pancreatic fat is a risk factor, above BMI, for the development of PaC. Furthermore, the quantification of this risk will be important to potentially identify people who may benefit from risk-modification or screening strategies.

1.2 PANCREATIC STEATOSIS AND PANCREATIC CANCER HYPOTHESIS

It is accepted that obesity is a risk factor for the development PaC and inflammation a putative pathway in the development of PaC. These statements are supported by epidemiological and preclinical data. However, the precise mechanisms of excess adiposity-driven PaC development are unknown. Emerging evidence from the cardiovascular and metabolic literature indicate that excess adiposity is associated with the infiltration of adipose tissue into organs.This leads to a local pro-inflammatory environment and there is evidence that associates intra-pancreatic fat deposition with pancreatic neoplasia development and progression, particularly in animal models. We hypothesise that this infiltration is important in the development of PaC. **Figure 1.1** outlines potential hypotheses for excess adiposity, chronic pancreatitis and PaC development. It is currently unknown if excess adiposity alone is enough to cause PaC (**hypothesis a**) or if pancreatic steatosis acts in conjunction with episodes of subclinical pancreatitis (**hypothesis b**).

Figure 1.1: Proposed hypothesis of pancreatic cancer development secondary to excess adiposity. Excess adiposity increases the risk of pancreatic cancer independent of clinical pancreatitis. Pancreatic steatosis causes pancreatic inflammation (subclinical chronic pancreatitis) that leads to PaC. It is unknown whether further inflammatory insult in the form of acute pancreatitis is required to induce inflammatory pathways that can lead to PaC.
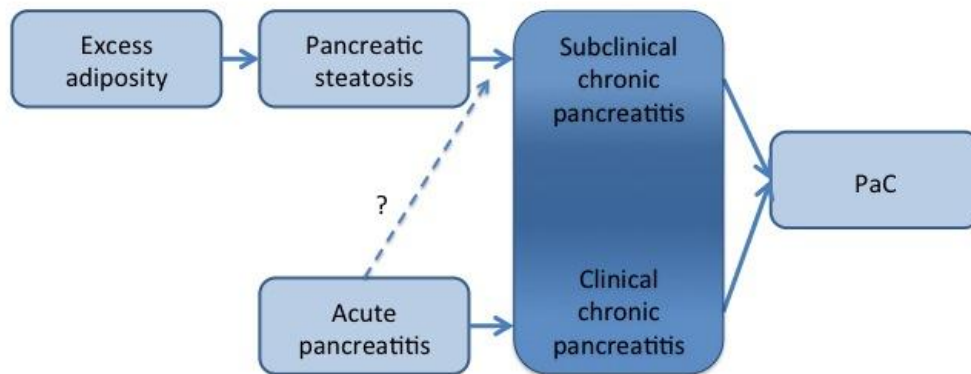
1.2.1 DIAGNOSIS AND QUANTIFICATION OF PANCREATIC STEATOSIS

The clinically significant or symptomatic degree of pancreatic fat is not known and there is no definition of the degree of fat infiltration that is considered 'steatosis'.[147] Some fat infiltration is considered normal[148] and quantification of pancreatic steatosis is dependent on the method of assessment. Human studies assessing pancreatic fat content are limited to histological analysis of specimens taken in the context of pancreatic disease or post-mortem specimens. Radiological assessment has been performed using semi-quantitative (USS and CT) or quantitative (MRS and CS-MR) techniques.

The amount of steatosis is quantified on histology either with a scoring system[149-151] or on a continuous scale expressed as a percentage of the histological slide.[113, 148, 149] Three authors, Gaujoux et al, Tranchart et al and Rebours et al used a subjective scoring system based on the presence of: no adipocytes, scattered adipocytes or numerous adipocytes in the intralobar and interlobar spaces.[143, 150, 151] However, it is known that in the context of hepatic steatosis, visual assessment frequently overestimates adipocyte presence. A more objective method is to calculate the proportion of the histological slide taken up by adipocytes as a percentage of the total.[152] Siasho et al and Hori et al calculated this percentage using Image Pro Plus Software[113] and WinROOF image analysis software[144] respectively but others have not described their technique.[23, 42] Although this technique provides a continuous scale, it may underestimate the overall fat content of the pancreas as

immunohistochemistry and electron microscopy studies in mice studies demonstrate that lipids accumulate within endocrine and exocrine cells prior to adipocyte infiltration.[153]

In patients without pancreatic disease in vivo assessment of pancreatic fat can be performed using radiological techniques. USS assesses the echogenicity of the pancreas and compares this with other organs, usually the spleen, with steatosis hyperechogeneic.[103] This method of assessment is subjective, does not provide a continuous scale of fat, and operator dependent. In addition, fibrosis also appears hyperechogeneic and visualisation of the pancreas is often difficult in obese patients limiting the use of this technique for research purposes.

CT scan techniques assess pancreatic density in Hounsfield Units (HU). Values similar to VAT indicate extensive pancreatic infiltration and mean density can be used to compare fat content with other areas of the body.[154] A calculated fat fraction can be obtained by calculating the proportion of the pancreas occupied by tissue with equal density in HU to VAT as a proportion of the total volume.[113] Although this technique may make comparisons between patients, it does not measure actual pancreatic fat content.

Chemical Shift Magnetic Resonance (CS-MR) and Magnetic Resonance Spectroscopy (MRS) are non-invasive imaging techniques. They are capable of quantitative assessment of PFF. However, their use is experimental, limited to small studies only and poorly validated. Considerable heterogeneity exists in scanning technique and expression of results. Some authors have validated MR techniques by scanning objects of known fat content, known as phantoms. Studies using MR techniques to measure pancreatic fat fraction are listed in **table 1.3**.

CS-MR compares the signal on opposed-phase and in-phase MR sequences. The ratio of signal attributable to fat to total signal is calculated and usually expressed as a percentage. Relative Signal Intensity Decrease (RSID) is a variation of this technique that compares the in-phase signals of the pancreas with the spleen to calculate fat relative to this organ. The spleen is chosen as an organ with very little adiposity.

Li et al assessed PFF in 126 healthy volunteers finding no difference in the PFF of the different areas of the pancreas (head, body, tail) and validated the accuracy of CS-MR using a phantom. Results demonstrated significant correlation between true and calculated fat using this method and the authors went on to devise a regression equation from which true PFF could be calculated.[155] Other studies[101, 156] using CS-MR have used techniques validated in the measurement of hepatic fat fraction without performing a phantom study. Although the values of PFF derived in these studies are of use for comparative purposes it is not clear if they truly represent PFF.

A single study by Lee et al compared CS-MR results with pancreatic tissue following resection. An RSID technique was used to calculate PFF and this was compared this with

histological assessment of PFF. They used linear regression to assess the relationship between histological findings and found a modest correlation (r^2= 0.560 p=0.013) between these measurements but did not assess accuracy.[149]

MRS is considered the gold standard for the non-invasive quantification of hepatic steatosis[156]. This technique separates signal from hydrogen atoms in different molecules (such as in water and fat) within the area targeted. PFF is calculated as the ratio of total lipid signal to water. It has been the focus of a number of small studies and differentiates between types of fat as well as the overall signal attributable to fat. Two authors[100, 107] have validated their MRS by including a second animal arm in their study from which pancreatic tissue was obtained. Lingvay et al and Hannukainen et al compared MRS PFF with biochemical PFF using rats and pigs respectively. Lingvay found an intra-class coefficient (ICC) of 0.91 and Hannukainen an r^2 of 0.876 indicating good correlation but not necessarily accuracy.[100, 107] Lingvay et al similarly reported good reproducibility of MRS (ICC 0.94) by repeating their investigation in a subset of patients at 2 weeks.

There remains a need for an accurate, reproducible and non-invasive technique to measure pancreatic fat fraction. None of the studies described have been used in the context of PaC risk. Only a single MR method (using RSID) has been validated with human pancreatic tissue.[149] No study has validated its results using both a phantom and human tissue.

Table 1.3: Magnetic resonance imaging studies quantifying pancreatic fat fraction

First author (year)	Study population	Study design	Findings
MR Spectroscopy			
Cho (2005)[157]	36 subjects	Patients undergoing pancreatic resections: 15 chronic pancreatitis 21 pancreatic carcinoma	Pathological diagnosis made on histology. No histological quantification of adipocyte presence. Spectra of chronic pancreatitis contained significantly less lipid than pancreatic cancer patients
Hannukainen (2010)[107]	16 human subjects 15 pigs	(a) 8 pairs of monzygotic male twins with discordant physical activity (mean age 25.8 years) (b) 15 pigs	No significant difference in pancreatic fat fraction between active and non-active twins. PFF associated with HFF, insulin sensitivity, adiponectin and glutamyltransferase concentrations. Good correlation observed between MRS-derived and biochemical measurement of pancreatic fat in pigs (r^2=0.876)
Lingvay (2009)[100]	79 human subjects 24 rats	Human population: 4 groups based on BMI and glucose tolerance using ADA guidelines Rat population: 12 lean and 12 obese males Reproducibility of MRS tested by duplicate measurement of MRS 1-2 weeks after initial measurement MRS of excised rat pancreata followed by biochemical determination of PFF.	Significant determinants of PFF on multivariate regression analysis- WHR, weight gain, OGTT ICC of 0.94 (95% CI, 0.88-0.97) for repeatability measures. Good correlation between rat MRS and biochemical PFF (r^2= 0.91) in rat arm of study
Ma (2011)[158]	56 human subjects	27 control and 29 patients with PaC. Spectral features of PaC and normal pancreas compared	No difference between FA or lipid content of head vs body-tail region in normal pancreas
Su (2012)[159]	32 human subjects	Characterisation of spectra at 3.0T using PRESS sequence	Metabolites characterised at 3.0 T included choline and lipids
Van der Zijl (2011)[106]	64 human subjects	Cross-sectional study comparing age and BMI matched individuals with NGT, IFG and IGT	Pancreatic fat content greater in IFG and IGT groups than NGT group.
Chemical Shift MR			
Li (2011)[155]	126 male human subject	BMI range: 18-25kg/m^2 Age range: 20-70	Human study: no significant difference between PFF of head/body/tail. PFF (mean 6.32%) of men aged 50-70 was twice as high as those aged 20-50 (2.8%) Phantom study: strong correlation (r^2=0.992) with measured and actual lipid content. Actual fat fraction estimation required conversion using a linear equation.
Wong (2014)[160]	Retrospective analysis of 685 healthy male subjects.	BMI: mean 22.7 kg/m^2 (±3.5%) Age: mean 48 (±10 years) PFF measured using IDEAL-reconstructed fat-only and water-only images. HFF measured using MRS	16.1% of the cohort had a fatty pancreas (defined here as >10.4%) this was not associated with HOMA-β are adjustment for liver fat and BMI. Serum ferritin, central obesity and hypertriglyceridaemia were independent factors associated with PFF
Rossi (2011)[105]	50 human subjects	12 lean subjects (BMI 22.85 ± 2 kg/m^2) 38 obese subjects (BMI 34.96 ± 4.12 kg/m^2) RSID technique used to estimate PFF.	Mean RSID of pancreatic lipid content 0.04 ± 0;06 PFF correlated with WC, serum triglycerides, adiponectin, daily fat intake but not BMI, age or weight.
Maggio (2012)[101]	49 adolescants	24 lean (BMI 18.9 ± 1.9 kg/m^2, mean age 13.2 ± 1.7 years) 25 obese (BMI 30.3 ± 5.4 kg/m^2, mean age 13.9 ± 1.2 years)	PFF higher in obese than lean subjects (4.8 ± 1.2, vs 3.6 ± 0.9) PFF associated with VAT, LFTS, triglycerides, HDL cholesterol, leptin.
Chemical Shift MR and MR Spectroscopy			
Sijens (2010)[156]	36 human subjects	36 volunteers, BMI mean 27.5kg/m^2 (range 20 to 42.9 kg/m^2) Dixon 2 point technique for CS-MR MRS with PRESS sequence	Moderate correlation between PFF and BMI (r^2=0.349), subcutaneous fat (r^2=0.442) and HFF (r^2=0.428) PFF and HFF of obese subjects higher than non-obese (not significant)
Schwenzer (2008)[161]	17 human subjects	Age: mean 50.4 (range 26-70 years) BMI unknown. Comparison of fat selective and spatial-spatial methods of assessing PFF	Mean PFF 8.8% (±5.7%) Good correlation between the 2 methods No difference between fat content in head/body/tail regions of pancreas. Accuracy influenced by T1 and T2* relaxation times of the tissue.

Pancreatic Fat Fraction (PFF), Hepatic Fat Fraction (HFF), American diabetes association (ADA), Intraclass correlation coefficient (ICC), waist-to-hip ratio (WHR), oral glucose tolerance test (OGTT), Fatty Acid (FA), body mass index (BMI), pancreatic cancer (PaC), relative signal intensity decrease (RSID), waist circumference (WC), normal glucose tolerance (NGT), impaired fasting glucose (IFG) and impaired glucose tolerance (IGT)

1.2.2 MODIFYING CANCER RISK

Unlike many risk factors for cancer development, excess adiposity is modifiable. Visceral, subcutaneous and ectopic adipose tissue depots are reduced by lifestyle changes such as dietary intervention or surgical intervention in the form of bariatric surgery. Furthermore, weight-loss has beneficial effects on metabolic pathways proposed to cause obesity-driven cancers. It is therefore logical to expect that cancer risk can be reduced with sustained weight loss. However, although studies examining this relationship in the epidemiological and bariatric literature have found a reduction in risk for some cancers, there is insufficient evidence to determine the effect of weight loss on pancreatic cancer incidence.

1.2.3 EPIDEMIOLOGICAL EVIDENCE OF WEIGHT LOSS AND CANCER RISK

The evidence describing the relationship between (non-surgical) intentional weight loss and cancer risk is limited. As sustained weight loss occurs only in a small proportion of participants in cohort studies, very large samples are required to be informative.

Analyses within epidemiological cohorts suggest that intentional weight loss (variably defined between 5 and 15 per cent) is associated with significant risk reductions for post-menopausal breast cancer effects on breast cancer risk.[162, 163] For gastrointestinal cancers, studies are limited to risk of colon cancer are less consistent. Thus, for example, Parker and Folsom,[164] defining intentional weight loss as greater than 16.4 per cent, reported a 9 per cent risk reduction for colon cancer among post-menopausal women, while Renehan and colleagues,[165] defining long-term weight loss as greater than 0.5 kg/year weight loss, found no significant risk reduction for colon or rectal cancer in either men or women.

For randomised controlled trials, large dietary intervention studies have assessed the impacts of low fat diets or fruit and vegetable enhancement of diets on breast cancer incidence or recurrence risk. While these trials were not designed specifically to reduce weight, the dietary interventions lead to weight differences between the randomised groups. In two of these trials – the Women's Intervention Nutrition Study[166] and the Women's Health Initiative trial[167] – weight reduction was associated with reduced breast cancer recurrence and incidence, respectively. There are few equivalent trials for gastrointestinal tumours. The exception is the Polyp Prevention trial,[168] which randomized 1,905 patients with colonoscopy-proven adenomas to a low-fat, high-fibre or their 'usual' diet. After 4 years, the trial found no differences in adenoma recurrence between intervention groups. In secondary analyses, while baseline obesity was associated with an increased risk of adenoma recurrence, weight gain or loss were not associated with recurrence, regardless of baseline BMI.

36

1.2.4 BARIATRIC SURGERY AND CANCER RISK

The beneficial metabolic effects of bariatric surgery are established; sustained mean weight reductions of 20 kg in morbidly obese patients (BMI ≥40 kg/m^2), which results in disease reversibility in over 70 per cent of patients with type-2 diabetes; reduced cardiovascular risk; and improved all-cause mortality.[169] In studies where there is an age-BMI matched control population, the beneficial effects extend to cancer risk; there are consistent inverse associations with the subsequent development of female sex-hormone sensitive cancers (notable endometrial and breast cancers) but not for male cancers.[170-177]

The majority of studies examining the association between bariatric surgery and cancer risk are retrospective with the notable exception of the Swedish Obesity Study (SOS), which is prospective. At first glance, the impact of bariatric surgery on cancer risk appears to be inconsistent. However, at deeper scrutiny, it becomes clear that these 'inconsistencies' can be readily explained by sex-specific associations and different study analytical methods. Thus, in studies where there is an age-BMI matched control population, there are consistent inverse associations with the subsequent development of female sex-hormone sensitive cancers (notable endometrial and breast cancers) but not for male cancers.[170] The extent of the 'cancer protective' effect of bariatric surgery in women is best exemplified in the SOS study: with greater than 10 year median follow-up, the risk reduction in women was 0.58 (95% CI 0.44–0.77), compared with that for men, 0.97 (95% CI 0. 62-1.52). The absence of effect in men might reflect small sample numbers (among series of bariatric surgery, only 14 to 35 percent of participants are male). Alternatively, as the median follow-up following surgery in these cohorts is approximately a decade, an explanation might be that the effects of weight reversal might take much longer to become apparent for other obesity-related cancers, such as colon, rectal, and kidney cancers, which are numerically more common in men.

Taken together, the current evidence supports a risk-reduction effect of bariatric surgery for post-menopausal breast and endometrial cancer. However, there is currently insufficient evidence whether or not bariatric surgery impacts favourably on gastrointestinal cancer incidence and studies so far are underpowered to detect any difference in pancreatic cancer incidence.

1.2.5 WEIGHT LOSS TRIALS AND PANCREATIC FAT FRACTION

Three studies have investigated the effect of weight loss interventions on PFF, two studies of dietary intervention and one of bariatric surgery have reported that weight loss leads to a reduction in PFF. Rossi et al measured the effect of a 500kcal below resting energy expenditure diet on PFF in 24 obese (mean BMI 35.4 kg/m^2) adults. Participants remained

on the diet until greater than 7% of initial weight was lost. A mean body weight decrease of 8.9% was associated with a 42.3% reduction in pancreatic fat.[178] Lim et al, studied eleven obese (mean BMI 33.6 kg/m^2) people with type II diabetes an 8-week severe calorie restricted diet (600kcal/day) and found a 15% reduction in body weight associated with a reduction in PFF of 23% and improvements in insulin suppression of hepatic glucose output.[179] Finally, a 44% reduction in PFF and 25% reduction in body weight was observed in 20 patients following bariatric surgical intervention.[180] Taken together, these studies are proof-of-principle that PFF can be modified.

1.3 RESEARCH AIMS

This literature review demonstrates that the influence of pancreatic steatosis on pancreatic cancer risk is unknown. I hypothesise that local fat infiltration may better quantify the risk attributable to excess adiposity than anthropometric markers such BMI. To test this hypothesis there is a need for a non-invasive test that can quantify pancreatic fat and derive accurate and reproducible values. MRS and CS-MR are promising techniques but are limited to a few studies only, and require further validation. We therefore aim to test the accuracy and reproducibility of these techniques in humans.

As a potential risk factor for pancreatic cancer development, intra-organ fat deposition presents a modifiable target. Yet, for intra-pancreatic fat, data that weight loss strategies reduce this adipose tissue depot are limited to three studies. Dietary interventions are varied and the ideal strategy to reduce ectopic depots is unknown. I intend to test the effect of dietary intervention on pancreatic fat

1.4 STUDY HYPOTHESIS AND AIMS

(i)   I can non-invasively quantify intra-pancreatic adipose tissue using MR imaging. This would be a potential fit-for-purpose biomarker for PaC risk.

(ii)   Dietary intervention and weight loss is associated with a reduction in pancreatic fat fraction; PFF is a modifiable potential risk factor for obesity-driven pancreatic cancer.

I therefore carried out the following studies:

1. To address whether pancreatic fat quantified by MR accurately measures in situ pancreatic fat in humans, I used the opportunity of patients undergoing surgical resection to compare digitally-derived histological assessment with CS-MR and MRS quantification.

2. To address reproducibility and explore relationships with other markers of excess adiposity, I performed repeated measurements of PFF in healthy volunteers.

3. Finally, to test reversibility I used the opportunity of a dietary intervention randomised controlled trial to determine if intra-pancreatic fat could be modified.

2   Methods

Preface

From January 2014 to October 2014, I ran two concurrent prospective clinical studies assessing whether MR imaging technology is 'fit for purpose' as a biomarker (BIDD/Assay Development Stage 3). These studies collectively make up the PanORAMA project (Pancreatic Cancer Predisposition, Obesity-Related Deposition Assessment using Magnetic Resonance ImAging), which was funded by Pancreatic Cancer UK. **Sections 2.2 and 2.3** *describe stages one and two of the PanORAMA project, respectively;* **sections 2.4 through 2.7** *describe techniques common to both studies. The two-stages (Results in Chapters 3 and 4, respectively) of the PanORAMA study were included under a single ethics application with approval granted by the Health Research Authority, National Research Ethics Service Committee North West, Greater Manchester West (13/NW/0814). The sponsor of this study was the University of Manchester. Local research approval was sought from the two NHS sites where research was conducted – The Pennine Acute Hospitals NHS Trust (where HPB surgery was undertaken) and The Christie NHS Foundation Trust, and its adjoining non-NHS University of Manchester imaging facility, the Wolfson Molecular Imaging Centre (where imaging and analyses were undertaken). Reproducibility results of the second stage of the PanORAMA project indicated the need for further development of our pancreatic MR standard operating procedures. A separate funding request from The University of Manchester to support MR development was made and an additional group of healthy volunteers recruited. Changes to the pancreatic MR methodology are outlined in* **section 2.8.**

The third part of the original research addressed the question of whether pancreatic fat was modifiable (Results in Chapter 5). We originally intended to 'piggy-back' onto an existing trial of weight reduction and prospectively assess changes in PFF. However, a unique opportunity arose to work in collaboration with the Genesis Breast Cancer Risk Reduction unit at the University Hospital South Manchester to design a trial that would utilise our MR imaging techniques. This part of the thesis was termed The BRRIDE-2 study (Breast Risk Reduction Intermittent Dietary Evaluation 2). This study is outlined in **sections 2.9.** *This study was part funded by Pancreatic Cancer UK, Help Against Liver Tumours (HALT) and Genesis Breast Cancer. The sponsor of this study was the University Hospital South Manchester and ethical approval granted by the Health Research Authority, National Research Ethics Service Committee South Central, Oxford B (14/SC/1097).*

2.1 THE PANORAMA PROJECT

2.1.1 BACKGROUND

There is a need for a non-invasive method of measuring pancreatic fat fraction (PFF). The two magnetic resonance (MR) imaging techniques, chemical shift magnetic resonance (CS-MR) and magnetic resonance spectroscopy (MRS) are described and validated as methods of measuring hepatic fat and have been used in a small number of studies addressing PFF, at the outset of this thesis, there were only six published studies on CS-MR and PFF and eight on MRS and PFF.[100, 101, 105-107, 155-161] My literature review identified a significant weakness of the published studies to-date, a lack of histological validation in human subjects. Therefore, to address the overall hypothesis I needed to validate the accuracy and reproducibility of these MR imaging techniques.

In the process of validating MR methods, there was the opportunity to ascertain the distribution of pancreatic fat within the pancreas in the healthy population and correlate this fat depot to other ectopic fat depots and anthropometric measures of excess adiposity. Establishing these relationships are important steps in our research program; we have hypothesised that for a given body mass index (BMI) there will be variation in PFF, and this variation may explain in part the variation in PaC risk with increasing BMI.

The PanORAMA project was therefore a two-stage project to (i) validate the accuracy of MRS and CS-MR through comparison with histological determination of intra-pancreatic fat and (ii) assess precision of MRS and CS-MR and explore relationships with other anthropometric measures. The study took place across three sites (schematic 1) the Pennine Acute Hospitals NHS Foundation Trust, the Christie NHS Foundation Trust and the Wolfson Molecular Imaging Centre (WMIC).

Figure 2.1: PanORAMA study locations

PanORAMA study setting
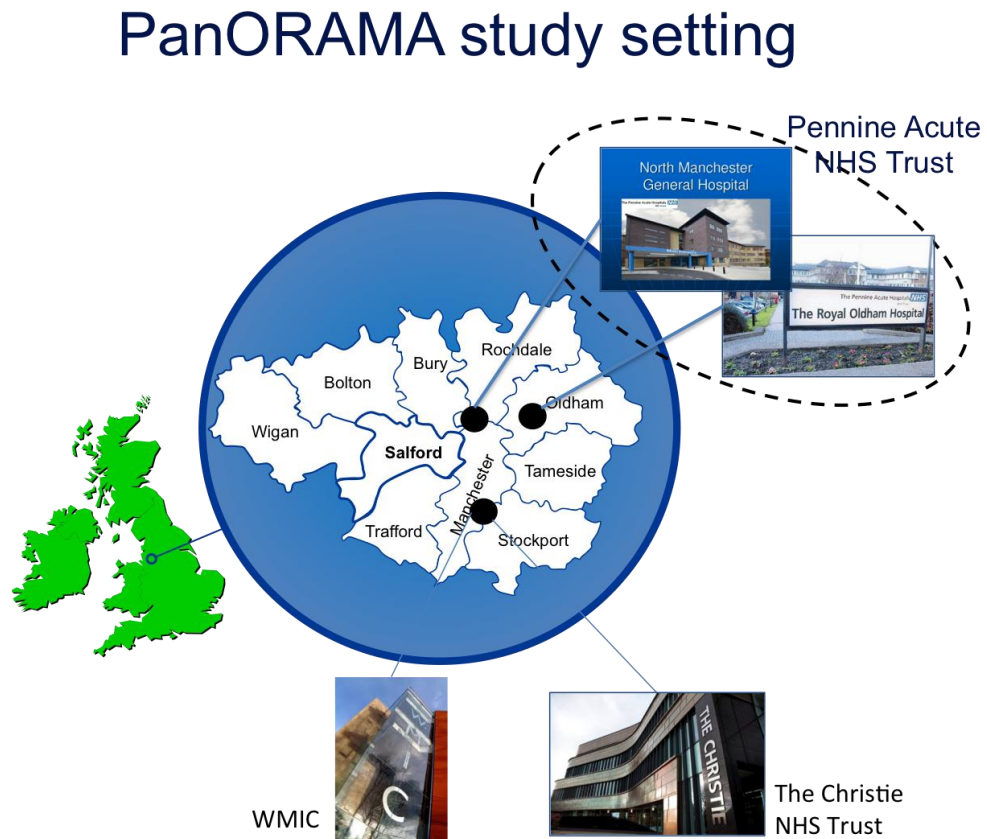
2.2 THE PANORAMA PROJECT STAGE 1 – STUDY DESIGN AND PATIENT RECRUITMENT

2.2.1 STUDY DESIGN

Patients were recruited from North Manchester General Hospital (NMGH), part of Pennine Acute Trust (PAT), at the time the largest HPB centre in Greater Manchester. There was a weekly multidisciplinary team (MDT) meeting at NMGH for patients with both benign and malignant disease of the liver, biliary system and pancreas and from here potential patients were identified. As a tertiary referral centre, patients attended NMGH from the Greater Manchester and Chesire catchment area.

In this first stage I recruited 12 individuals undergoing pancreatic resection for either benign or malignant disease. Patients discussed at the MDT who required pancreatic resection were invited to attend the outpatient clinic to see a consultant surgeon and to discuss treatment options. It is here that I met with these patients and discussed the project. At this point, the project aims were outlined to patients and they were provided with a patient information sheet. I asked their permission to contact them at least 48 hours after this initial meeting to give patients time to consider the study and recorded this permission. At least 48 hours after the initial meeting we contacted each patient by telephone to discuss further any

questions and if they wished to be part of the study to arrange a time and date prior to their surgery for them to attend the WMIC.

Patients were consented on their arrival to the WMIC. In addition to the MR scan sequences I took anthropometric measurements of waist and hip circumference, height and weight. The CS-MR and MRS sequences were performed in one sitting which took approximately 45 minutes.

Following surgery, pancreatic tissue taken as part of the procedure was sent to The Royal Oldham Hospital (ROH) site for histopathological analysis. Eight digital images of Haematoxylin and Eosin stained pancreas at 2x magnification were taken, anonymised and sent to the student. Using digital histology techniques translated from this groups previous work with hepatic fat, fat content was quantified as a proportion of the overall slide.[181] MR scans were assessed under the supervision of Professor Stephen Williams for PFF and HFF (MRS and CS-MR), pancreatic lipid types (MRS) and visceral and subcutaneous fat volumes.

Figure 2.2: Study flow diagram for PanORAMA stage 1
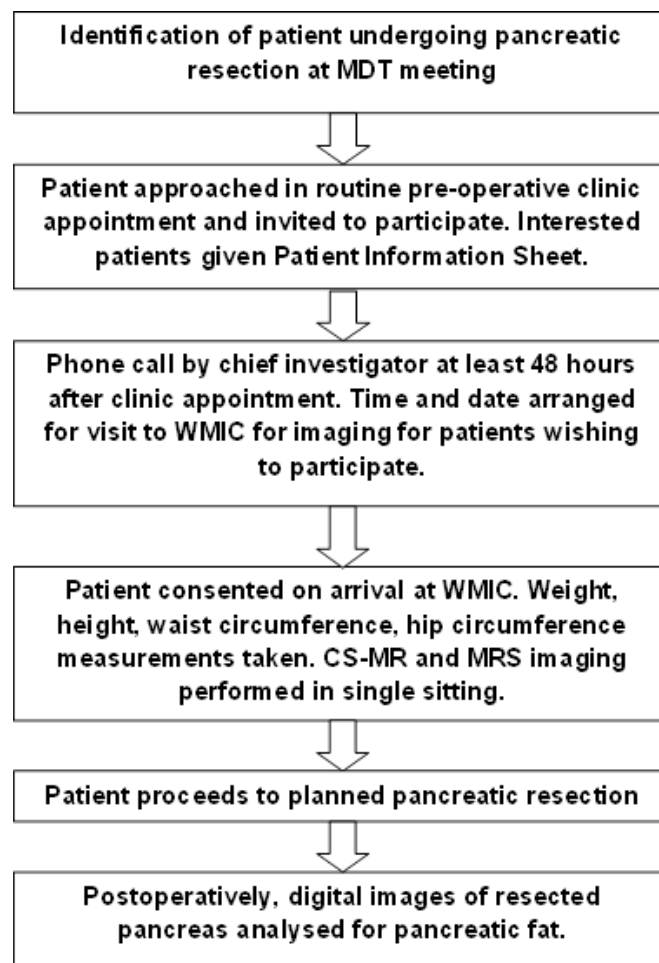
2.2.2 INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria

1. Patients must be able to receive and understand verbal and written information regarding the study and give written, informed consent.
2. Patients due to undergo surgical resection of part, or all, of the pancreas gland.

Exclusion criteria

1. Persons under 18 years of age.
2. Conditions in which the supine position and breath holds required for MR scanning are not possible.
3. Persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs.
4. Persons with contraindications to MR imaging- presence of cardiac pacemaker/artificial heart valve/aneurysm clips/metallic fragments in eyes/cochlear implants

2.2.3 POWER CALCULATION

Formal power calculations were not applicable in this setting. Estimated numbers of patients were pragmatic based upon annual throughput at NMGH – approximately 50 pancreatic resections per year. Taking in to account the likely variability in referrals, theatre listings and patient choice, we aimed to recruit up to 15 evaluable participants.

2.2.4 JUSTIFICATION FOR CHOSEN APPROACH

There remains a need for an accurate, reproducible and non-invasive technique to measure pancreatic fat fraction. The literature review found only one MR method validated with human pancreatic tissue. As the pancreas gland is a relatively inaccessible organ and cannot be biopsied in the same manner as the liver, the setting of pancreatic resections is an ideal opportunity to validate CS-MR and MRS. It is accepted that pathology will alter PFF so the results of this stage of the study do not reflect normal pancreatic fat content.

As the long-term aim of this research stream is to investigate intra-pancreatic fat deposition as a potentially more sensitive biomarker of pancreatic cancer development, this strategy would involve the screening of the healthy population. Any screening test therefore needs to be non-harmful. For this reason, magnetic resonance imaging techniques are an ideal choice as they are non-invasive and have no known side effects. Importantly, unlike CT methods, they do not deliver a radiation dose.

2.3 THE PANORAMA PROJECT STAGE 2 – STUDY DESIGN AND PARTICIPANT RECRUITMENT

2.3.1 STUDY DESIGN

In this second stage, we recruited 15 individuals identified as having no known current or prior pancreatic disease. These patients were recruited as volunteers from the Christie NHS Foundation Trust via word of mouth. After an initial discussion, volunteers were provided with a participant information sheet and then contacted at least 48 hours later to confirm that they still wished to be part of the study. If they wished to be part of the study we arranged a time and date for the research MR scan at WMIC.

At the WMIC, the CS-MR and MRS were performed and repeated in one sitting, this took approximately 60 minutes. Additionally, we performed measurements of waist and hip circumference, height and weight. The MR scans were assessed by the student under the supervision of Professor Stephen Williams for pancreatic fat fraction and pancreatic lipid types as well as hepatic fat fraction, visceral and subcutaneous fat volumes.

Figure 2.3: Study flow diagram for PanORAMA stage 2
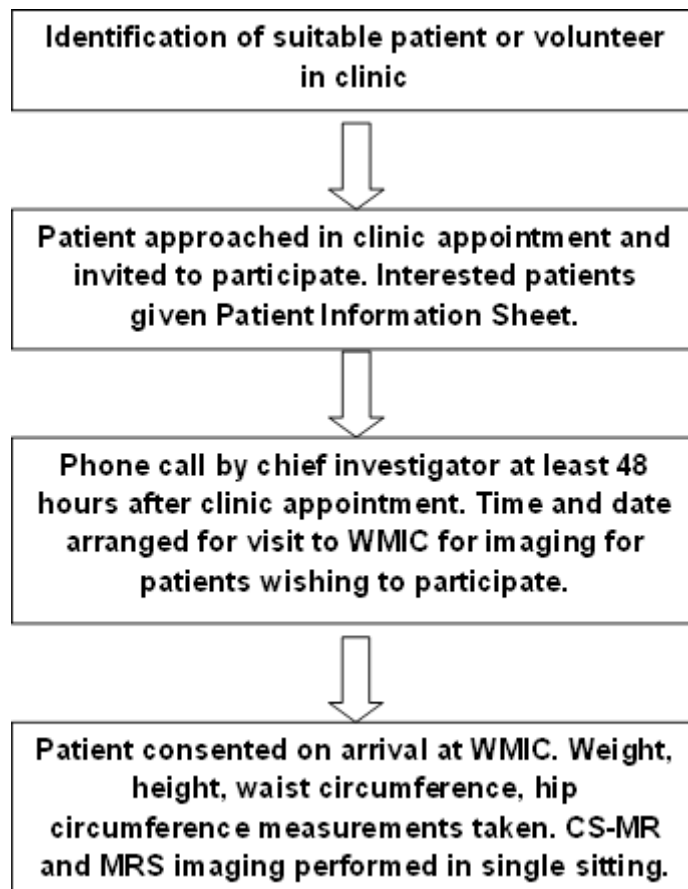
2.3.2 INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria

1. Volunteers must be able to receive and understand verbal and written information regarding the study and give written, informed consent.
2. Volunteers without current or a history of pancreatic disease.

Exclusion criteria

1. Persons under 18 years of age.
2. Conditions in which the supine position and breath holds required for MR scanning are not possible.
3. Persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs.
4. Persons with contraindications to MR imaging- i.e. presence of cardiac pacemaker/artificial heart valve/aneurysm clips/metallic fragments in eyes/cochlear implants

2.3.3 POWER CALCULATION

Formal power calculations are not applicable in this setting.

2.4 PANORAMA PROJECT – ^{1}H MRS

2.4.1 THEORY OF H^1 MRS

Single-voxel MRS is the gold-standard for ectopic fat quantification. It yields a precise spectrum of chemical composition within one voxel. MRS relies on chemical shift, which refers to differences in the Larmor frequencies of water and fat protons. Water protons from (-OH) hydroxyl groups are characterized by a spectral peak at 4.69 ppm (parts-per-million). In contrast, the predominant protons of triglycerides are from the (-CH2) methylene groups. Due to different chemical environments surrounding the protons (oxygen in water versus carbon in triglycerides), methylene protons have a slightly lower resonant frequency. The frequency separation between water and the methylene fat peak is linearly proportional to the field strength, such that larger chemical shift separations are achieved with increasing field strengths. At 1.5 Teslas and body temperature, the water-fat chemical shift is approximately 220 Hz.

2.4.2 ^{1}H MRS PROTOCOL

Previous research by Frahm et al indicated that the STEAM (STimulated Echo Acquisition Mode) localisation method provided optimum MRS outputs.[182] Voxel volume and dimensions were adjusted to fit the dimensions of the intended organ; in the pancreas a voxel of 1000

mm^3 volume was used with dimensions 10mm x 10mm x 10mm in the pancreatic head and 5mm x 20mm x 10mm in the body; in the liver a 15mm x 15mm x 15mm voxel was used.

2.4.3 ANALYSIS OF ^{1}H MRS OUTPUTS

The majority of proton signal from hepatic lipids is from the methylene functional groups of saturated fatty acids (including 14:0, 16:0, 18:0, 20:0, and 22:0) and the "saturated" methylene components of monounsaturated fatty acids and poly unsaturated fatty acids (PUFAs). Together these account for the significantly greater signal amplitude of the methylene resonance at approximately 1.3 ppm. *In vivo* HFF is determined as the percentage of this bulk methylene resonance to water corrected for T_2 effects.

Combined spectra analysis

Spectral data were post-processed by magnetic resonance user interface software (jMRUI version 5.1 alpha, EU Project).[183] The Java Magnetic Resonance User Interface was opened and after Fourier transformation and manual phasing of the spectra, the water peak was identified and its chemical shift set to 4.69 ppm.

Spectra were analysed using the AMARES routine in jMRUI[184] First, each spectra was manually phased using the zero order phase tool: the centre of each peak and approximate line-width were identified manually before analysis by the program. The amplitude of the peak is returned by the routine and corresponds to the total amount of that signal in the spectrum.

Post spectral analysis

Fat fraction was calculated as lipid signal as a proportion of the entire signal so that:

$$\text{Fat fraction} = \text{lipid} / (\text{water} + \text{lipid})$$

This needs to be corrected for the greater attenuation of the water signal compared to fat as a result of the acquisition sequence. Taking average values for the water and fat relaxation times T2 (determined experimentally for each subject, but averaged across all subjects) of 69 ms for fat, and 58 ms for water. The correction is:

$$\exp(TE[1/T2w - 1/T2f])$$

Where T2w and T2f are the relaxation times for water and fat and TE is the 'spin-echo' time for the sequence (10 ms in this case).

2.5 PANORAMA PROJECT – CHEMICAL SHIFT MR

2.5.1 THEORY OF CHEMICAL SHIFT MR

By controlling the echo time when data is acquired after RF excitation, the net detected MRI signal can comprise either of water and fat in-phase (IP=W+F, aligned, phase=0 degrees) or out-of-phase (OP=W-F, anti-aligned, phase=180 degrees). By using this two-point (IP and

OP) approach, separated water and fat images could be obtained by image algebra. By reconstructing separated water and fat images, a subsequent percent fat fraction map (fat:water ratio) can be computed, which would facilitate measurement of fat accumulation in organs on a voxel-by-voxel basis. Intuitively, one can realize that for a voxel containing only water or fat, its net signal will be the same on IP and OP acquisitions as one component's signal will be zero. In contrast, a voxel containing both water and fat will have different signals from the two acquisitions.

2.5.2 CHEMICAL SHIFT MR PROTOCOL

Images were obtained using the following parameters: Echo Time (TE) 2.3ms, Repetition Time (TR) 150ms. This TR was chosen to minimise T2 effects, but has to be set against reasonable scanning and breath holds. We used a multi-echo spin sequence with 9 breath holds of 8 seconds each to acquire images.

2.5.3 ANALYSIS OF CHEMICAL SHIFT MR IMAGES

For each chemical shift MR image, we measured signal intensity at TEs of 2.3ms (OP), 4.6ms(IP1), 9.2ms (IP2) in three areas of the liver and the pancreatic head, body and tail regions using OsiriX software. Regions of interest (ROI) of 1cm in diameter were drawn in each part of the pancreas and average signal intensity within these ROIs recorded.

For patients in PanORAMA stage-one we measured signal intensity at the resection margin, identified using the superior mesenteric vein/ splenic vein confluence and superior mesenteric artery. Here, ROIs were determined by individual participant pancreatic size. Pancreatic head signal intensity was always measured to the right of the inferior mesenteric vein. For the liver, ROIs of 4cm in diameter were drawn in Couinaud segments III, V and VIII of the liver (chosen because of their central location, to reduce motion artefacts) and were placed away from major vessels and ducts. Where it was not possible to use segments III, V and VIII, the nearest suitable segment was chosen.

Fat fraction was derived using the standard formula ([IP-OP] / [2IP]). The in-phase needs to be corrected for T2 decay therefore the IP signal was calculated using acquisitions at 4.6ms (IP1) and 9.2ms (IP2) using the following formula.

$$IP = IP1*(SQRT(IP1/IP2))$$

2.5.4 THE PANORAMA PROJECT – MEASUREMENT OF VISCERAL AND SUBCUTANEOUS FAT

T1 weighted axial images of the abdomen from symphysis pubis to the upper border of the liver were obtained using the following parameters: TE 15ms, TR 450ms, slice thickness 20mm.

Post-acquisition analysis was performed using OsiriX software a free-to-download DICOM viewing application.[185] First, the L5/S1 intervertebral disc was identified and images cranially to the diaphragm were selected. Using the draw tool, visceral and subcutaneous areas were manually defined. A histogram of signal intensity of adipose tissue was obtained by drawing a ROI of 4cm x 4cm in the subcutaneous adipose tissue and the signal relating to fat identified from this.

Using the grow region tool, voxels corresponding to the chosen signal intensity were automatically identified by the software. The 3D grow tool was used to calculate the volume of fat across all chosen slices. By selecting out the visceral or subcutaneous areas on each slice, volume of adipose tissue in each of these compartments was calculated.

2.5.5 PANORAMA PROJECT – SEMI-AUTOMATED MEASUREMENT OF INTRA-PANCREATIC FAT

Due to its difficulty in being assessed, pancreatic fat is not routinely assessed and therefore no standard way of measuring pancreatic fat is agreed upon. As discussed in the literature review, the amount of fat deposition has been quantified on histology either with a scoring system[149-151] or on a continuous scale expressed as a percentage of the histological slide.[113, 148, 149] It is known that in the context of hepatic steatosis, visual assessment frequently overestimates adipocyte percentage. We therefore aimed to translate our experience with hepatic fat quantification to pancreatic fat quantification.[181]
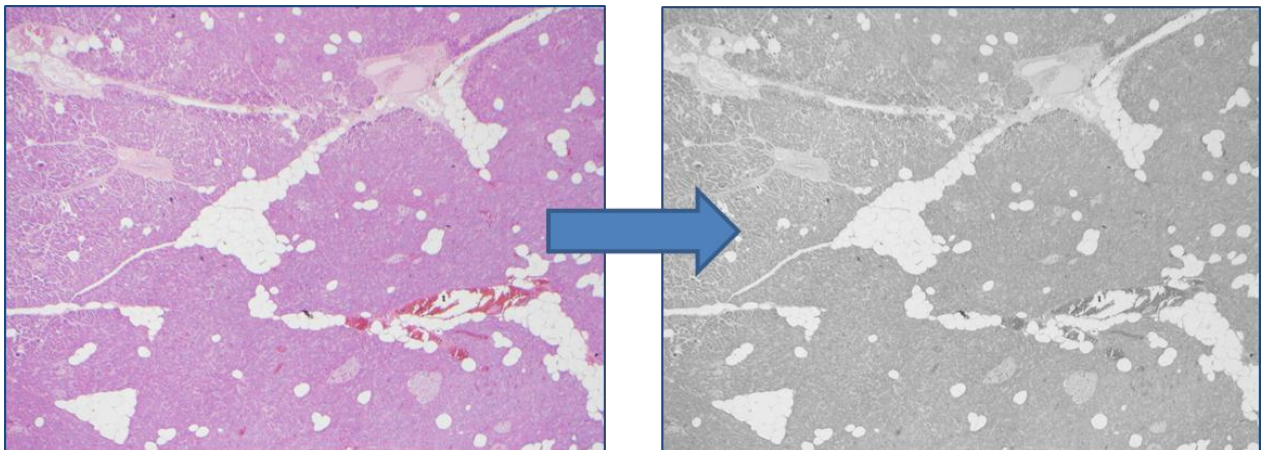
Figure 2.4: Image of tissue from the pancreatic resection margin, stained with Haematoxyin and Eosin (left) and converted into a grey-scale image (right).

2.5.6 HISTOLOGICAL QUANTIFICATION METHODOLOGY

For patients in the PANORAMA study, 8 digital images of routine H&E slides of tissue at the pancreatic resection margin were taken by a single consultant hepatobiliary pathologist (Dr Madhu Rao) at x10 magnification and maximum resolution. Dr Rao chose sections judged to be representative of the whole specimen. The digital histology images were assessed in the department of medical illustration at The Christie NHS Foundation Trust using Adobe Photoshop software.

Each photograph was converted to a greyscale image and the levels adjusted to increase the contrast between the grey parenchyma and the white fat (figure 2.4). The image was magnified by zooming in and the magic wand tool was used to grab areas of fat, leaving behind normal parenchyma. The PFF percentage was determined by counting the number of pixels highlighted by the magic wand and dividing by the total number in the original unhighlighted image using the histogram tool. Individual percentages were recorded and a mean PFF was derived for each patient from the 8 digital images.

2.6 PANORAMA EXTENSION PROJECT

In Stage 2.1 of the PanORAMA project, assessment of reproducibility demonstrated significant variability for repeated measurements of pancreatic MRS. A number of potential improvements to our pancreatic MRS protocol were identified and a successful application for development scanning time was made to the University of Manchester for a Magnetic Resonance Imaging Facilities (MRIF) grant. Ten healthy volunteers were recruited to undergo repeated pancreatic MRS additionally, in preparation for the BRRIDE-2 project, this was an opportunity to test reproducibility of hepatic MRS.

We considered that a likely source of variability in our reproducibility measurements was movement of the pancreas due to diaphragm movement during respiration. An MRS voxel is placed based on planning images. If the MRS data acquisition occurs in a different phase of respiration to the planning sequence then data are acquired from a different anatomical position to what was intended. Typically for the pancreas this would be the surrounding visceral fat. Four changes to the pancreatic MRS protocol were therefore made to test if we could improve reproducibility:

1. Reduced voxel depth (to 5mm x 40mm x 5mm from 10mm x 20mm x 5mm) while maintaining voxel volume to reduce potential overlap into surrounding visceral fat.
2. Control of the phase of respiration in which planning sequences are taken and MRS data acquired. This was achieved using breath holds for planning sequences and respiratory triggered MRS sequences.

3. Additional oblique pancreas planning sequence to improve pancreas voxel placement
4. Dynamic collection MRS data. Sixteen MRS spectra are acquired and averaged for every STEAM sequence. To ensure the quality of this data we will collect each spectra individually and inspect them in an additional post-processing step.

2.6.1 STUDY DESIGN

Ten healthy volunteers were recruited to undergo repeated (same-day) measurements of pancreatic head and body MRS and liver MRS. These volunteers were recruited from the Christies NHS Foundation Trust and via word-of mouth. They were given a participant information sheet and at least 48 hours before being booked for an imaging appointment.

2.7 THE BRRIDE-2 PROJECT

A unique opportunity arose during my project to test the hypothesis that pancreatic fat could be modified. My original intention had been to test this hypothesis by collaborating with an already running dietary intervention trial, recruiting participants to undergo MR measurement of intra-pancreatic fat at the start and end of dietary intervention. Instead, during discussions with my collaborators, there was interest in utilising our experience with MR quantification of ectopic fat to develop an improved design of a randomised controlled trial to compare the effect of different dietary interventions on ectopic fat stores.

For my collaborators (Harvie, Higham, Howell), reduction in intra-hepatic fat was the primary outcome measure. This team are interested in obesity and post-menopausal breast cancer risk. Insulin resistance is a proposed mechanism for this increased risk with intra-hepatic fat considered a key driver of insulin resistance.[186, 187] The study was therefore designed with intra-hepatic fat reduction as the primary outcome measure. Reductions in intra-pancreatic fat are explorative due to the paucity of previous studies addressing this question so intra-pancreatic fat reduction was a secondary outcome within this study.

The BRRIDE-2 study (Breast Risk Reduction Intermittent Dietary Evaluation 2), a randomised controlled clinical trial comparing the effect of intermittent calorie restriction with daily calorie restriction on hepatic and pancreatic adipose stores and insulin resistance, ran between January 2015 and October 2015. This study was part funded by Pancreatic Cancer UK, Help Against Liver Tumours (HALT) and Genesis Breast Cancer. The sponsor of this study was the University Hospital South Manchester and ethical approval granted by the Health Research Authority, National Research Ethics Service Committee South Central, Oxford B (14/SC/1097). During the study it became apparent that the drop-out rate was higher than anticipated so an amendment application granted to allow the recruitment of two extra participants.

2.8   THE BRRIDE-2 PROJECT – OVERVIEW

Hypothesis: Energy restricted diets cause reductions in hepatic and visceral fat and reduced insulin resistance and hence reduced cancer risk. Intermittent dieting is an increasingly popular method of dieting[188] which involves short spells of severe restriction and spells of normal intake. We have shown that intermittent dieting leads to a greater reduction in insulin resistance than daily dieting.

We hypothesise that an intermittent energy restricted diet will lead to a greater reduction in hepatic fat compared to an isoenergetic daily moderate energy restricted diet. This study will define the metabolic effects of intermittent compared to standard dieting and inform its value as a potential cancer risk reduction strategy.

Design: A randomised controlled trial to compare the effects of two eight-week energy restricted diets - (i) an Intermittent Energy Restriction (IER) diet versus (ii) a Daily Energy Restriction (DER) diet - on magnetic resonance (MR) imaging quantified body, hepatic and pancreatic fat deposition distribution, and insulin resistance in obese women

Outcome measures:

Primary endpoints are
(i)   Quantity of intrahepatic fat (hepatic fat fraction HFF) of hepatic fat, determined using phase-contrast magnetic resonance (CS-MR) and MR spectroscopy (MRS).
(ii)   Insulin resistance determined using Oral Glucose Tolerance Testing (OGTT).

Secondary outcome are changes in:
(i)   Quantity of pancreatic fat (PFF)
(ii)   MR-derived fat stores; visceral, subcutaneous, and intramyocellular fat.

Sample size: 14 participants in each arm (total 28) randomised 1:1. The sample size was powered to detect a 15% difference in the reduction of hepatic fat fraction (HFF) between the 2 diet groups and to include an estimated 20% drop-out rate.

Study Population: Participants were obese pre-menopausal women, aged 25- 50 years, with a Body Mass Index (BMI) of between 30 and 45 Kg/m^2, and non-smokers. Women were at increased risk of breast cancer and were identified within the regional Family History Clinic at the Genesis Breast Cancer Prevention Centre, University Hospital South Manchester.

2.8.1 THE BRRIDE-2 PROJECT – BACKGROUND

Chronic conditions characterised by hyperinsulinaemia, such as obesity and type 2 diabetes are established risk factors for post-menopausal breast cancer incidence[189] and additionally carry an adverse prognosis in pre and post-menopausal women following initial treatment for breast cancer.[190, 191] The biological mechanisms underpinning this risk and progression are incompletely understood, but insulin resistance is hypothesised to be an important mediator. Other systemic or endocrine adiposity-related systems, such as sex hormones (oestrogen, testosterone), inflammatory cytokines and adipokines (leptin, adiponectin) in part regulated through insulin resistance are also relevant.[94, 189, 192] Ectopic fat deposition in the liver "intra-hepatic fat" causes local inflammation, and in tandem with visceral fat, is considered a key driver of systemic insulin resistance.[193] Thus, hepatic fat may be linked to all obesity-driven cancers including pancreatic.[194-196]

Obesity is a modifiable risk factor and maintained modest weight reduction (5% or greater) in both pre and post-menopausal years has been shown to reduce breast cancer risk after the menopause by 28-40%.[162, 163] Dietary intervention to reduce weight is therefore a potential cancer risk reduction strategy. An ideal dietary strategy would be easy to adhere to, preferentially reduce visceral and ectopic fat volumes whilst preserving lean mass, and resting energy expenditure (REE) and achieve reductions in insulin resistance and preferential balance of adipokines as surrogates for cancer risk reduction.

Intermittent energy restriction (IER) is a novel dietary approach which has been shown to be comparable,[188] or easier for people to follow than daily energy restriction (DER).[197] In animal models, IER is superior or equivalent to DER with respect to reduction in breast cancer risk,[198] increased survival in established prostate[199] and pancreatic cancer[125], reduction of cardiovascular and cerebrovascular disease[200] dementia[201] and increased longevity.[202]

The IER diet includes two consecutive days of severe energy restriction and five days of normal dietary intake. In a randomised controlled trial comparing 6 months of IER with DER, it has demonstrated greater reductions in insulin levels that with comparable weight loss with DER.[197] The mechanism underlying this is unknown. Reduced insulin resistance after 6 months of IER was observed during the five normal eating days of IER but 25% further reductions in both insulin resistance and serum triacylglycerol (TAG) were seen during fasting days.[188, 197]

Accumulation of hepatic and intramyocellular TAG is proposed as a common pathway leading to impaired systemic insulin signalling and insulin resistance.[186] Excess hepatic TAG, known as hepatic steatosis, is a primary determinant of insulin resistance independent of body mass index (BMI), per-cent body fat, and visceral fat volume.[186] The

liver stores fat as TAG in response to increased circulating fatty acids and glucose and releases fat in the form of very low density lipoproteins (VLDL).[203] The IER diet involves 2 days of severe restriction (70%) instead of the standard approach of a modest daily 25% restriction. Studies in female C57BL/6J mice suggest ectopic and visceral fat stores are mobilised with IER regimens which includes restricted spells of 70% or greater but not with a daily 25% energy restriction.[204]

Hypothesis 1: I hypothesise that the greater reductions in insulin resistance seen during normal eating days with an IER diet compared with DER for a given energy deficit / weight loss may be due to an overall greater reduction in hepatic fat.

Hypothesis 2: I hypothesise that there are further reductions in hepatic fat and insulin resistance during the 2-consecutive fasting days each week on the IER diet.[203, 205]

I randomised obese women to either an IER or DER diet for a period of eight weeks. Primary endpoints are (i) quantity of intrahepatic fat (intra-hepatic fraction) determined using non-invasive non-ionising radiation imaging, namely MRS, and (ii) insulin resistance determined using HOMA and OGTT.

To test hypothesis (i), I compared differences in primary endpoints between IER and DER at the end of 8 weeks taking account of between-person variability at baseline. Trial assessments occured five days after fasting days in the IER group to assess the effects of IER vs. DER on the five normal eating days of the week away from any acute effects of the fasting days.

To test hypothesis (ii), I determined and compared the primary and secondary endpoints determined on the morning immediately after the 2-day restriction to a corresponding day of the week in the DER group during the seventh week following the IER or DER diets.

2.8.2 THE BRRIDE-2 PROJECT – STUDY LOCATION AND PARTICIPANT RECRUITMENT

This project was undertaken under the umbrella of the Manchester Cancer Research Centre (MCRC), involving the Christies NHS Foundation Trust, the adjoining University of Manchester Wolfson Molecular Imaging Centre (WMIC); and the Genesis Prevention Centre, University Hospital South Manchester (UHSM). It ran between January 2015 and October 2015. In brief, recruitment, dietary intervention advice, measurement of resting energy expenditure and anthropometric measures were performed at the Genesis Prevention Centre, magnetic resonance imaging data acquired at WMIC and insulin sensitivity data at the Christie NHS Foundation Trust.

2.8.3 BRRIDE-2 RESEARCH TEAM

The BRRIDE-2 trial bought together collaborators from three different sites. The dietician team were based at the Univeristy Hospital South Manchester (lead Dr Michelle Harvie) and have previously completed multiple studies on dietary intervention and markers of breast cancer risk.[188, 197] The team here were responsible for the day-to-day running of the trial including correspondence with participants, maintaining the trial master file and data handling. MR imaging was performed by the radiographers at the Wolfson Molecular Imaging Centre, the University of Manchester and analysis supported by Professor Stephen Williams. At The Christie NHS Foundation Trust, Dr Claire Higham and the specialist research nurse team (Rowan Challis, Grace Ensah) supported the oral glucose tolerance testing.

Figure 2.5: BRRIDE-2 study locations
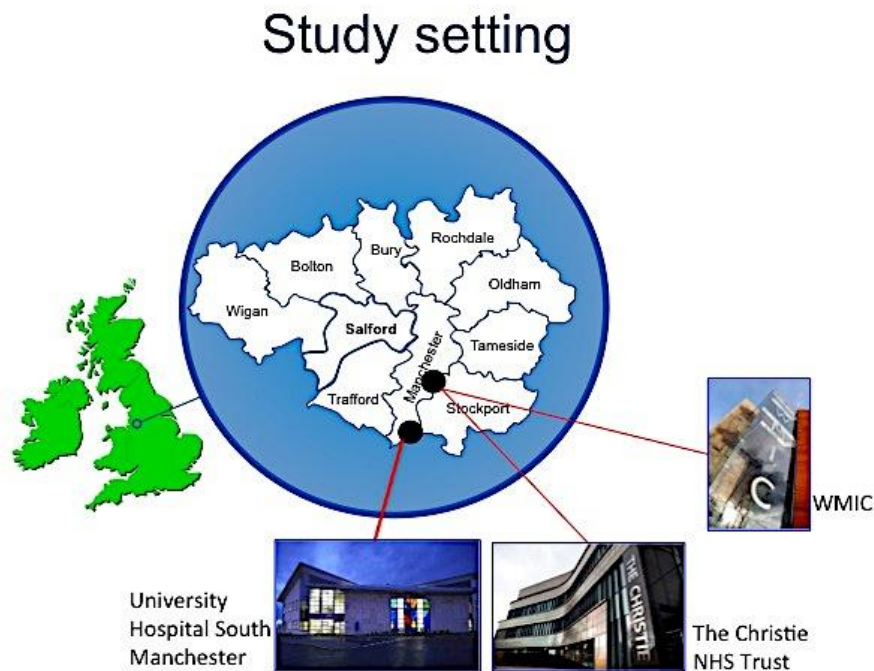
2.8.4 RECRUITMENT AND PARTICIPANT PATHWAY

Participants were women at increased risk of breast cancer recruited from the regional breast cancer Family History clinic at the Genesis Prevention Centre UHSM, or from recruited from the staff at The Christie NHS Foundation Trust, University Hospital South Manchester or University of Manchester. The family history clinic (lead clinicians: Professor Tony Howell; Professor Gareth Evans) has an established database of participants at

increased risk of breast cancer who attend for annual mammography and had indicated that they were happy to be approached about research trials. Additionally, the study was advertised via internal communication systems at UHSM and the Christies NHS Foundation Trust.

Interested participants were recruited by providing a participant information sheet to clinic attendees when they attended for their annual mammogram, by mailing an invitation to potentially eligible women who have previously expressed an interest in being part of dietary trials, after expressions of interest from staff at the study hospital's. Interested participants were invited to discuss the trial face to face or over the phone with the BRRIDE-2 research team. At this point, they were screened for eligibility and suitability and any further questions answered.

Interested participants were asked to maintain their current dietary intake in a 7-day food diary or to record their intake for 7 days using the on line dietary assessment tool (MyFood24 www.myfood24.org). They were asked to maintain their current activity levels and record these by completing the 7 day International Physical Activity Questionnaire (IPAQ) long version.[206] They were booked an appointment for MR imaging and insulin investigations to be performed at the Wolfson Molecular Imaging Centre (WMIC) and the Christies NHS Foundation Trust once they have completed these baseline 7 day diet and exercise assessments.

Fully informed consent for the study was taken on arrival at the WMIC. Participants were asked to arrive having fasted at the WMIC for MR imaging from 21:00 the evening previously. After MR imaging they underwent measurements of insulin and glucose at the Christies NHS Trust, a neighbouring site. Blood was also taken for biochemical markers of cancer risk.

Following this visit and within the same week, they attended the Genesis Prevention Centre Unit at UHSM to see the research dietitian. Here, they had their REE measured (Fitmate GS portable desktop indirect calorimeter (Cosmed, Rome Italy), baseline measures of body fat and fat free mass (bioelectrical impedance [Tanita 180] Tokyo, Japan) and anthropometrics (weight, height, waist and bust circumference) and were randomised onto one of the two diet groups and given comprehensive advice and materials to enable them to follow their allocated dietary regime. They were asked to commence their allocated diet that week. Participants were contacted by telephone by their allocated dietitian one week later to check that they had started the diet, their understanding of the diet and to provide any trouble shooting advice.

We asked participants to attend the Genesis Prevention Centre Unit in week 2, 4, 6 for a face-to-face review and weigh in with their allocated dietitian. Their allocated dietitian phoned them in weeks 3, 5 and 7 for a 20-minute conversation to discuss adherence and

any problems with the diet. At the mid-point of the study we arranged the time and date of week 7 and 8 attendances at the WMIC and the Christie NHS Trust.

In week 7 all participants in both diet groups will attend the WMIC for MR imaging and The Christie NHS Foundation Trust for insulin and glucose measurements, and biochemical tests. Participants in the IER group attended on the morning after the 48-hour restriction.

All participants attended WMIC in week 8 of their diet for a final MR scan. Following this, biochemical markers of cancer risk, insulin and glucose were repeated at the Christie NHS Trust. This test was timed when the IER group are at least 4 days after their restricted diet days to avoid any acute effects of the restricted days on fat stores and insulin sensitivity. Later that week participants re-attended UHSM to reassess REE, body fat, fat free mass, anthropometrics and to review the week eight 7-day food records and IPAQ activity questionnaire. Participants were given moving on diet and exercise advice (IER or DER) and offered two further monthly review appointments with the trial dietitian if they wish.

2.9 THE BRRIDE-2 PROJECT – INCLUSION AND EXCLUSION CRITERIA

Participant Population
Inclusion Criteria
1. Premenopausal aged >25-50 years
2. Body mass index 30-45 kg/m^2.
3. Non-smoker
4. Sedentary (< 40 minutes moderate exercise per week)

Exclusion Criteria
1. Weight greater than 125kg
2. Already successfully losing weight.
3. Pregnant or planning pregnancy over next 5 months
4. Currently Breast feeding
5. Eating disorder, depression or alcoholism
6. Alcohol intake greater than 10g of ethanol (10 units) per week[207].
7. Co-morbidity i.e. Non-Alcoholic Fatty Liver Disease, diabetes, viral hepatitis, fibrosis, Human Immunodeficiency Virus, coeliac disease.
8. Drug use current or within the past 6 months affecting hepatic fat content i.e. insulin, oral contraceptives, tamoxifen, statins, amiodarone, methotrexate, corticosteroids.
9. Previous or current history of cancer.
10. Following an incompatible therapeutic diet.
11. Contraindication to MR imaging (e.g. pacemaker)

2.10 THE BRRIDE-2 PROJECT – DIETARY INTERVENTION

2.10.1 DIETARY INTERVENTION.

Participants were randomised to one of two 25% energy restricted diets. These provide 75% of their estimated energy requirements for a two-month weight loss period. Baseline energy requirements for each participant were determined using indirect calorimetry (Fitmate GS portable desktop indirect calorimeter (Cosmed, Rome Italy)

2.10.2 INTERMITTENT ENERGY RESTRICTION

A low carbohydrate energy restricted diet (600 kcal, <50g carbohydrate, 50 g protein day) (70% energy restriction) for two consecutive days and~1900 kcal Mediterranean diet for the remaining five days of the week. Each of the two low carbohydrate 600 kcal energy restricted days included; ~ 300g of lean protein foods e.g. lean meat, fish, eggs, tofu, quorn, textured vegetable protein, three portions of low fat dairy foods, five portions of low carbohydrate vegetables, one portion of low carbohydrate fruit and two pints of low energy drinks. The five unrestricted days were based on a Mediterranean diet which provides 30% energy from fat (15% MUFA, 8% PUFA, 7% saturated) 25% energy from protein and 45% from low glycaemic load carbohydrate and allows up to 10 units of alcohol per week. Research to date has shown that the majority of IER dieters chose to diet on the same 2 days each week.[188] Forming habits is key for compliance with diet interventions[208]. We encouraged this in the current study to ensure compliance and standardisation of the intermittent diet.

2.10.3 DAILY ENERGY RESTRICTION

A daily 25% energy restricted Mediterranean diet (~1500kcal/day) for seven days/week. The Mediterranean diet provides 30% energy from fat (15% MUFA, 8% PUFA, 7% saturated), 25% energy from protein and 45% from low glycaemic load carbohydrate and allows up to 10 units of alcohol per week.

      The IER and DER diets were matched for energy and macronutrient composition. Both diets provide 45% energy from carbohydrate, 25% from protein and 30% from fat (15% MUFA, 7% saturated fat and 8% PUFA) and allow up to 10 units of alcohol per week. I deliberately recruited sedentary individuals to the trial. All participants were advised to maintain current low activity levels, and not to become more active for the duration of the study (8 weeks). The study was designed to examine the effect of diet on hepatic and ectopic fat. I therefore needed to ensure that participants did not become more active which

could confound any observed changes in fat stores.[209] Therefore, I assessed 7-day activity levels at baseline and week 8 using the validated IPAQ questionnaire.[206]

2.10.4 ADVICE, SUPPORT AND MONITORING IN BOTH DIET GROUPS

Foods eaten on the IER and DER diets were self-selected by the patients and not provided by the study team.

The IER and DER groups received clear instructions of how to follow their allocated diet in a face to face dietary consultation with one of the trial dietitians (45- 60 minute appointment) at the Genesis Prevention Centre. Both groups received comprehensive written instructions of how to follow the diets at home, including recommended portion sizes and recipes and suggested meal plans. Both groups received appropriate behavioural techniques to promote adherence to diets i.e. self-monitoring of diet and weight, and goal setting.

Participants were contacted by telephone by their allocated dietitian one week after starting to check that they have started the diet, their understanding of the diet and to provide any trouble shooting advice. Both groups attended the Genesis Prevention Centre Unit in week 2, 4, 6 for a face-to-face review and weigh in with their allocated dietitian with phone calls in week 3, 5 and 7 to discuss adherence and any problems with the diet. Both groups were asked to record 7-day food records either as written paper diaries or on line using My Food 24. This allowed the team to assess adherence to their allocated diet and served as an important tool to enhance compliance. The IER group were also asked to record their adherence to the 2-day IER each week on a special trial diary sheet.

2.10.5 JUSTIFICATION OF APPROACH

This trial tested the effects of intermittent vs. daily energy restriction on HFF. Hepatic fat fraction is reduced by energy restriction but is also modifiable by dietary composition. Greater hepatic fat loss is observed in a carbohydrate restricted energy restriction versus a higher carbohydrate isocaloric energy restriction,[210] and by increasing the amount of monounsaturated fatty acids (MUFA) in an isoenergetic diet.[211] I therefore ensured that the IER and DER diets are matched for macronutrient composition i.e. the percentage of energy obtained from carbohydrate, protein, monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fat, and only differed by having either an intermittent (2 consecutive days) or daily mode of energy restriction.

The overall macronutrient composition of the IER and DER diets in the trial was 25% energy from protein, 30% fat (15% MUFA, 8% PUFA, 7% saturated fat) and 41- 45% energy from carbohydrates and 0-4% from alcohol. The trial was designed to minimise fluctuations

in habitual alcohol intake when following the IER and DER diets which could independently influence changes in hepatic fat. The trial only included low habitual drinkers i.e. 0 – 10 units / week. The test IER and DER diets will include 0-5 units of alcohol / week, hence a maximum reduction of 5 units of alcohol per week. Prospective studies have shown that fluctuations (increases or decreases) of alcohol intake of 10g / day do not influence hepatic fat.[212]

Dietary intake of energy, fat (MUFA, PUFA and saturated fat), carbohydrate, protein, fibre and alcohol was assessed on a weekly basis throughout the trial from self-reported daily food records inputted by participants on a daily basis to an on line dietary assessment tool (MyFood24 www.myfood24.org) or from paper food diaries. Weekly physical activity level (International physical activity questionnaire long version [IPAQ] was assessed at baseline and week 8 of the trial to confirm that participants remain sedentary throughout the trial.[206]

2.10.6 RATIONALE FOR MEASURING LEAN BODY MASS AND RESTING ENERGY EXPENDITURE

Optimum weight loss diets should maximise loss of body fat and preserve LBM. Maintenance of LBM with weight loss is important for physical function and maintenance of REE, since LBM is a primary determinant of REE. Weight loss with DER diets leads to some loss of LBM mainly via decreased protein synthesis and a failure to reduce proteolysis and reductions in hydration of LBM, which can reduce muscle function.[213, 214]

Loss of LBM with DER is a function of dietary protein content, percentage body fat and subject exercise levels. LBM loss increases with the degree of DER. Typically DER regimens of < 500 kcal, 500 - 1000 kcal and > 1000 kcal/d respectively result in 60, 35 and 20% of weight to be lost as lean body mass.[215] The specific effects of IER on muscle mass is an important unresolved question. Proponents of IER diets claim that IER may preserve LBM as an adaptive-response to allow our Paleolithic ancestors to survive spells of food shortage. This would require IER to maintain protein synthesis and to reduce muscle proteolysis perhaps by preserving mitochondria function and structural integrity, increased mitochondrial biogenesis, reduction of oxidative stress and favourable modulation of apoptotic and autophagic signaling pathways.[216]

On the other hand the spells of severe restriction with IER may be problematic if they evoke decreased protein synthesis and increased proteolysis. There are limited data of the effects of IER on protein turnover, however a recent short term study of alternate day fasting (ADF) in healthy men found that ADF did not reduce proteolysis and reduced lower mTOR phosphorylation with a potential decrease in protein synthesis suggesting short term ADF may not specifically preserve muscle mass[217]. Clinical trials of longer periods of ADF and

IER diets (3 – 6 months) however suggest short spells of severe energy restrictions with IER do not appear to evoke losses of LBM seen with an equivalent daily DER. Previous intermittent diets based on alternate days of 500 kcal and adlib feeding (alternate day fasting) have reported minimal loss of LBM with 10% of weight lost as fat[218]. No trials have directly compared the percentage of weight which is lost as fat with ADF vs. DER. Our recent IER group lost 20% of their weight loss as LBM vs. 30% with DER (P<0.05). The slightly increased average protein intake in the IER group 80g vs. 70 g / day may be a factor in the preservation of LBM with our IER. Our previous trial found IER and DER had an equivalent loss of weight as fat 79 (24) vs. 79 (26)% (P =0.99) when both diets provided 70g protein[188]. Both the IER and DER groups in the present study were advised to consume comparable amounts of protein (1g/kg ideal body weight) therefore this trial was able to specifically assess the effect of the intermittent vs. daily pattern of low energy diets on lean body mass.

Preserving LBM is important for helping to maintain REE, however weight loss and DER evoke rapid reductions in REE and dietary induced thermogenesis (the component energy expenditure above resting value which results from the digestion, absorption and processing of nutrients) even alongside preservation of LBM.[219] This adaptive down-regulation of metabolism is linked to reduced circulating levels of leptin and thyroid hormones and a resultant blunting of the sympathetic nervous activity which typically lead to an overall 10% reduction in total energy expenditure within two-weeks of starting a daily 25% energy restriction.[220] This adaptive drop in REE is problematic for the dieter, and typically means actual weight loss is 30% less than predicted from their energy prescription and hence can be a major demotivating factor.[221] Reduced REE appears to persist in the post-dieting phase which could compromise weight loss maintenance and hence long term disease prevention.[222]

It is possible that the short spells of restriction each week with IER does not evoke the same adaptive reductions in REE as DER. Reductions in leptin are still likely to occur within the 1-2 days of respective energy [223] and carbohydrate restriction [224, 225] however these spells are limited to 2 days per week with IER. Two short-term studies of ADF in normal weight subjects have reported 3 - 5% [226] reductions in REE on the morning after normal feeding days of ADF which suggest that an adaptive response is occurring with short term ADF.

2.10.7 THE BRRIDE-2 PROJECT – MR IMAGING

MR imaging was performed in the same manner as the PanORAMA project. Changes to the PanORAMA protocol made in the extension project were used for pancreatic MRS acquisition.

2.10.8 POWER CALCULATION

While the primary endpoint of the overall thesis is intra-pancreatic fat quantification, for BRRIDE2, the primary endpoint was hepatic fat fraction as this is a key determinant of insulin resistance – a hypothesised key mediator of obesity-driven cancer development.[186, 187] The primary comparative question was between the IER diet and DER groups over a 2 months period. The sample size of 13 subjects per group was estimated [by the UHSM statistician, Julia Morris) to detect a difference of 15% in the reduction of hepatic fat fraction between the two different diets, assuming an estimated 20% drop-out rate. Calculations assume a two-sided t-test with estimated standard deviation of 10% and the conventional 5% significance level. As previously mentioned, during the study it became apparent that our drop-out rate was greater than anticipated. An amendment was made to the protocol and approved by the Health Research Authority, National Research Ethics Service Committee South Central, Oxford B (14/SC/1097) to allow us to recruit 2 extra participants.

2.10.9 STATISTICAL ANALYSIS

The primary analysis was performed on a per-protocol basis. i.e. participants who complied with dietary intervention and achieved weight loss $\geq$ 5%, and undertook the imaging and insulin resistance testing. This reflected that this was a study to investigate the mechanistic effect of the diet, rather than a pragmatic comparison of 2 diets.

As it is reasonable to expect that the starting weight, BMI, intra-hepatic fat, intra-pancreatic fat, and HOMA-IR influences final measurement, it would not have been appropriate to perform a direct comparison of week eight final measurements by group using either a t-test of Mann Whitney U. Instead I used a mixed-effects model repeated measures (MMRM) approach to test for the impact of treatment on the changes in measures of weight, BMI, HFF, PFF and HOMA-IR with time, while retaining within person correlation. In this setting, this approach was preferred (over ANOVA) as the variance of the data was unequal. I used a 2 level model, to include time and person as levels, and with treatment as a covariate. I set up an initiator and then fit the model using adaptive quadrature, using the STATA `gllamm` command.

2.10.10 ROLE OF THE FUNDERS

The funders had no input into study design, analysis or data interpretation.

3 VALIDATION OF MRS AND CS-MR - ACCURACY

Accuracy is defined as the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found experimentally.

At the start of this thesis (in 2012/13), two key findings of the literature review were (i) the absence of a human study that validated MRS and CS-MR techniques by comparing results with histological assessment of pancreatic fat; and (ii) the lack of a 'best' imaging method to quantify pancreatic fat on a continuous scale. Stage-one of the PanORAMA study was conceived to address these research gaps. In clinical practice, tissue suitable for the histological assessment of pancreatic fat is obtained only following pancreatic operations. In this prospective clinical study, I extended our group's experience with digital quantification of hepatic fat deposition to that of the pancreas, and related these measurements with preoperative MRS and CS-MR measurements of pancreatic fat. Additionally, this was an opportunity to obtain some exploratory MRS characterisation on pancreatic masses and relationships between pancreatic fat, hepatic fat and other anthropometric indices in this patient group.

The PanORAMA project aims to determine if pancreatic MRS and CS-MR techniques are 'fit-for-purpose' to measure intra-pancreatic fat, as a potential susceptibility biomarker for pancreatic cancer development. There is an established procedure to validate a biomarker as 'fit-for-purpose' developed predominantly for blood-borne assay assessment, and applicable here.[227, 228] Validation of a biomarker follows systematic steps to assure that the technique used is reliable to perform its task.[229] This rigorous process has been adopted by the imaging biomarker community.[230] Thus, the development of imaging biomarkers has to undergo the same assessment process, from discovery, through verification, validation and qualification before they can be used in clinical routine.[230]

Stage-one of the PanORAMA project deals directly with one aspect of biomarker method validation, determination of accuracy. In stage-two of the PanORAMA study (Chapter 4), I turn to assessing precision (reproducibility). Accuracy is defined as the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found experimentally. Prior to embarking on assessment of a biomarker method target values and acceptance limits should be agreed upon. Internationally recognized performance standards for chemical assay assessment are established; a study of both precision (% coefficient of variation, or CV) and accuracy (mean % deviation, or bias, from nominal concentration) is required. Precision and accuracy of

repeat analyses of the validation samples are expected to vary by less than 15%, except at the lower limit of quantitation, where 20% is allowable.[227] However, although fixed performance standards are necessary, by their nature they are arbitrary and do not necessarily relate to the intrinsic properties of the assay under investigation or, more importantly, its purpose.[230] If the biomarker with its newly established performance criteria can deliver to expectations, it is deemed fit for that purpose and valid. If not, then it cannot be deemed either fit for the specified purpose or valid.

Here, I used histologically determined intra-pancreatic fat as my reference standard for intra-pancreatic fat the gold-standard and compared this measure with MRS and CS-MR determined intra-pancreatic fat.

3.1 STAGE 1 PANORAMA METHODOLOGY SUMMARY

The technical methodology used to acquire CS-MR and MRS data from the pancreas is covered fully in chapter 2. To summarise, data from three MRS voxels placed at the pancreatic transection margin (figures 3.1 and 3.2, voxel A) and three CS-MR regions of interest (ROIs) were obtained in patients undergoing pancreatic resections and compared with histological assessment of intra-pancreatic fat from transection margin of the resected pancreatic specimen (figure 3.3). Additionally, we collected data on intra-hepatic fat and other anthropometric measures of excess adiposity and MRS data from pancreatic masses (figure 3.1, voxels B and C).
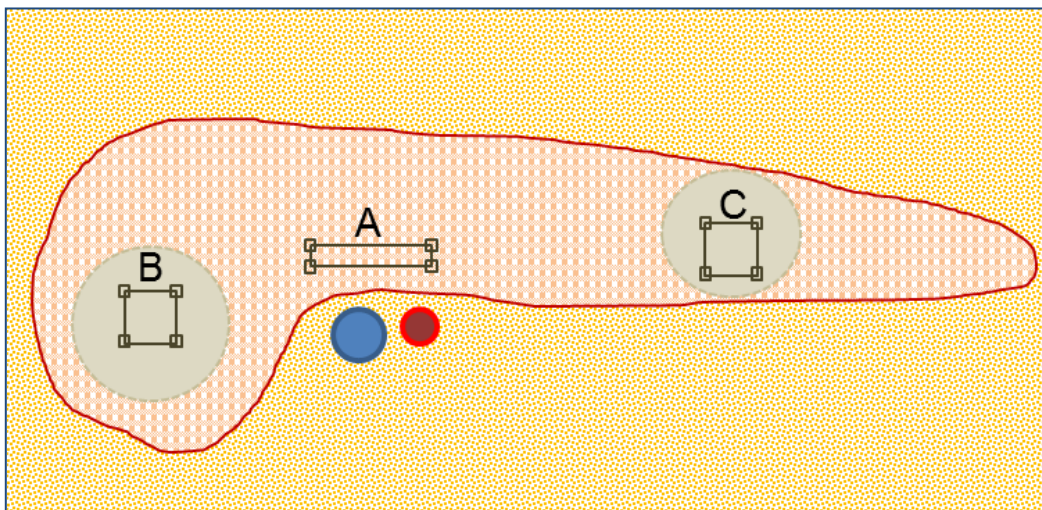
Figure 3.1: Pancreatic schematic demonstrating voxel (black rectangles) positioning for MRS data acquisition in stage 1. Data were acquired from the pancreatic transection margin (A) defined as the area immediately anterior to the superior mesenteric artery (red) and vein (blue) and from pancreatic masses of the head (B) or tail (C).
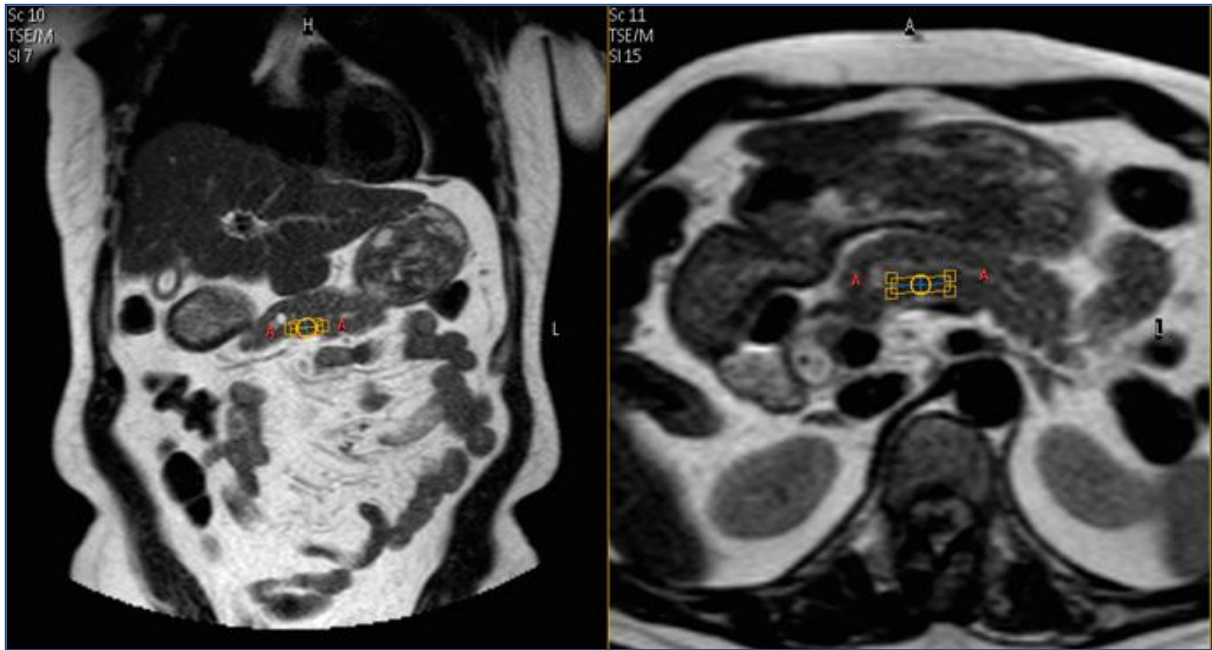
Figure 3.2: Screen shot from MR computer. Voxel (orange rectangle) positioning at the transection margin is achieved using coronal (left) and axial (right) images. MRS data is then acquired from this volume.

3.2 STAGE 1 PanORAMA PATIENT CHARACTERISTICS

Over a 10-month period (January 2014 to October 2014), 12 patients undergoing pancreatic resection for either benign or malignant disease were recruited onto the PanORAMA stage-one study. The median age was 67 years, nine were male and three female. All 12 patients underwent MR imaging of the pancreas for measurement of intra-pancreatic and intra-hepatic fat using CS-MR and MRS sequences. Of these 12 patients, two were unresectable at intra-operative assessment and therefore no pancreatic tissue was obtained, leaving a total of 10 with histology. Of these 10 patients, three had distal cholangiocarcinoma, two perimampullary adenocarcinoma, two pancreatic pseudocysts, one inflammatory biliary stricture, one neuroendocrine tumour and one pancreatic ductal adenocarcinoma. These patients are summarised in table 3.1.

		Frequency
Operation	Pylorus-preserving pancreatico duodenectomy	8
	Distal pancreatectomy	2
	Inoperable	2
Histology	Distal cholangiocarcinoma	3
	Peri-amupllary adenocarcinoma	2
	Pancreatic pseudocyst	2
	Inflammatory biliary stricture	1
	Neuroendocrine tumour	1
	Pancreatic ductal adenocarcinoma	1

Table 3.1: Summary histology and operative characteristics of Stage I PanORAMA patients

	Median (range)
Age (years)	67 (45 – 82)
BMI (kg/m^2)	23.4 (19.3 – 30.7)
Waist circumference	87.5 (83 – 106)
Waist-to-hip ratio	0.93 (0.74 – 0.97)
Histologically determined pancreatic fat %	2.2 (0.3 – 9.3)
MRS measured intra-pancreatic fat (%)	4.8 (0.3 – 10.9)
CS-MR measured intra-pancreatic fat (%)	3.6 (0.1 – 10.4)
MRS measured intrahepatic fat (%)	1.9 (0.1 – 9.1)
Visceral adipose tissue (cm^3)	98 (48 – 165)
Subcutaneous adipose tissue (cm^3)	113 (70 – 190)

Table 3.2: MR and anthropometric characteristics of Stage I PanORAMA patients

Table 3.2 shows the anthropometric characteristics of patients in PanORAMA stage 1. No significant correlations were seen between histologically determined pancreatic fat and other measures of excess adiposity; age (rho= -0.35, p=0.32), BMI (rho= 0.456, p= 0.19), waist circumference (rho= -0.15, p=0.67), waist-to-hip ratio (rho=-0.41, p=0.23) or MRS determined hepatic fat (rho= -0.44, p= 0.21).

3.3 HISTOLOGICAL DETERMINATION OF INTRA-PANCREATIC FAT

Mean histologically determined intra-pancreatic fat in this patient group ranged from 0.3% to 9.1% (figure 3.3).
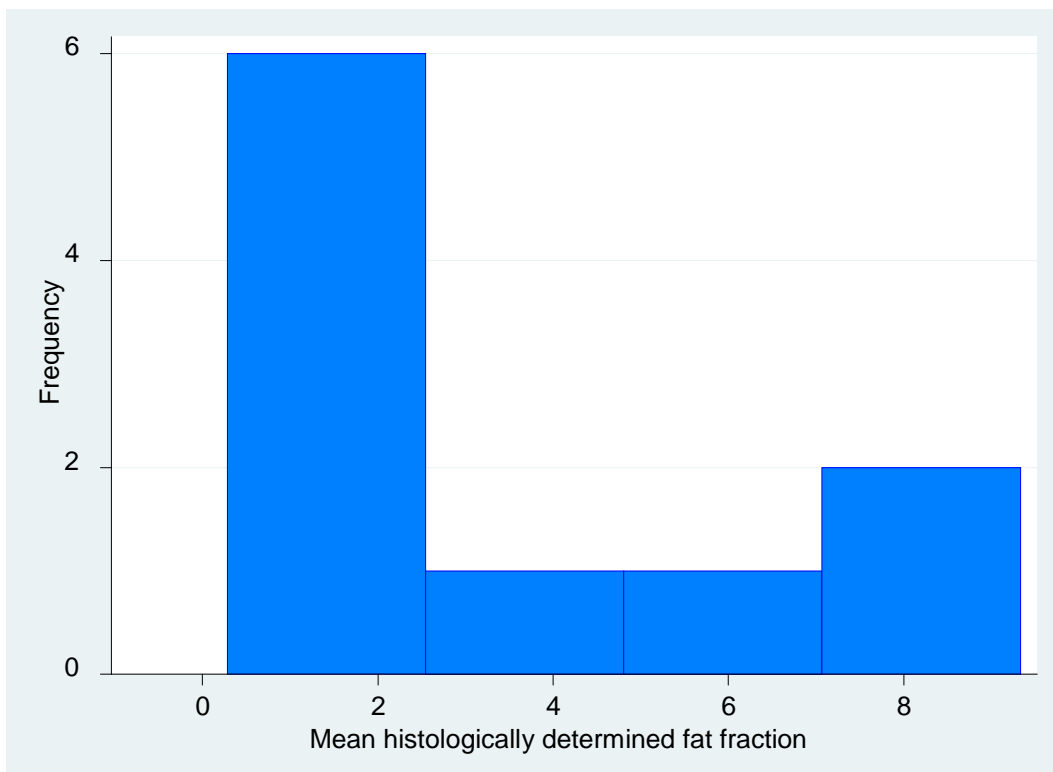
Figure 3.3: Frequency of mean histological determined fat fraction. The collected intra-operative samples represented a range of intra-pancreatic fat. This range is similar to that seen in the healthy population.
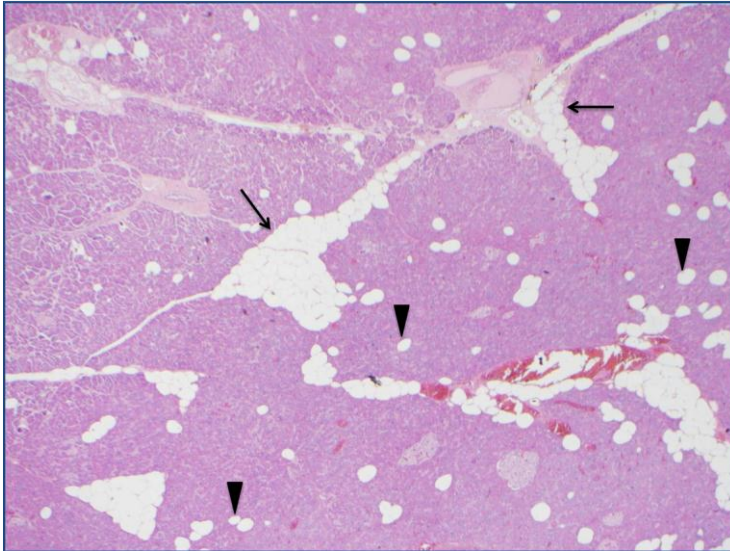
A:
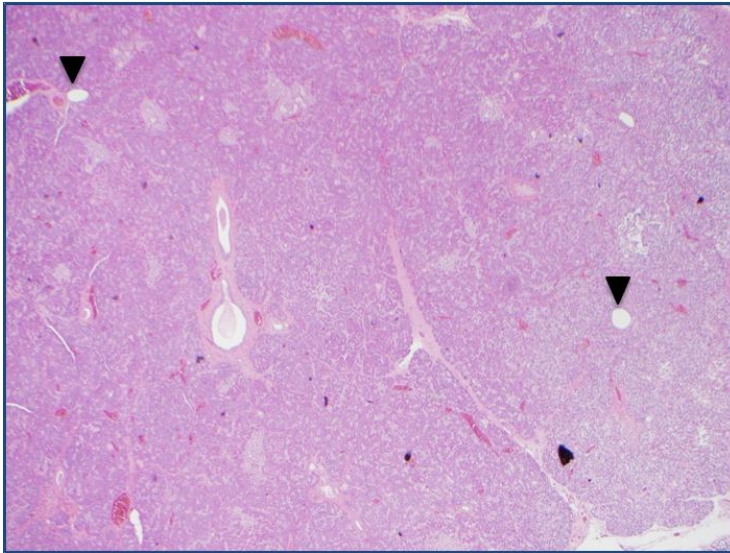
B:

Figure 3.4: Figure 3.4 A and B are individual slides at the extremes the range of intra-pancreatic fat observed in this study. Pancreatic fat was observed to be distributed within lobules and between lobules. Pancreatic tissue stained with haematoxylin and eosin. Image A: 9.5% pancreatic fat. Intra-lobular fatty infiltration (arrow heads) and extra-lobular fatty infiltration (arrows) is seen. Image B: 0.2% pancreatic fat, only sparse intra-lobular fat (arrow heads) is seen.

3.4 INTER-RATER VARIABILITY OF MRS AND CS-MR

To assess reproducibility of MRS and CS-MR techniques inter-rater variability was assessed using the concordance correlation coefficient (Stata command: concord). Deevia Kotecha (3rd year medical student, 2014) independently analysed 90 MRS spectra and CS-MR images. Correlation was strong: the concordance correlation coefficient for inter-rater variability was 0.98 and 0.91 respectively.

3.5 ACCURACY

Accuracy was assessed using Bland-Altman plots (levels of agreement), coefficients of variation and concordance correlations.

3.5.1 PANCREATIC MRS

Visual assessment using Bland-Altman plots (figure 3.5) show moderate levels of agreement between MRS measurement and histological measurement of pancreatic fat.
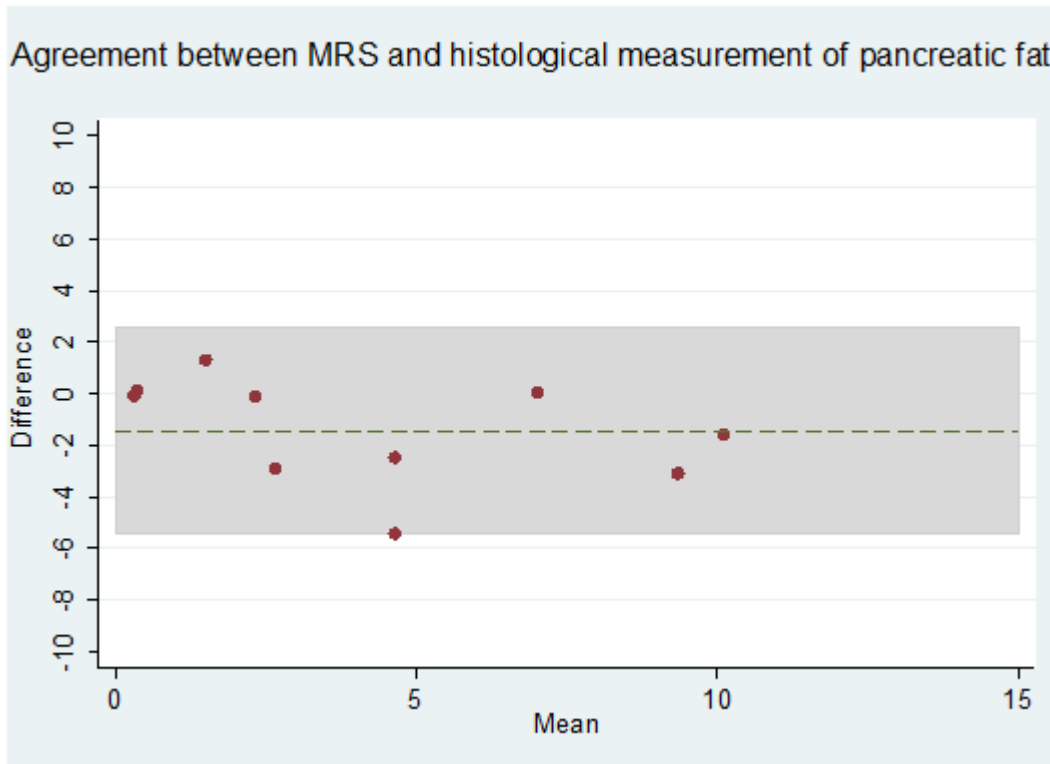
Figure 3.5: Bland Altman plot of agreement between MRS and histological measurement of pancreatic fat. The grey area represents 95% confidence interval and the green dashed line the average difference between measurements. This graph demonstrates that MRS measurement of pancreatic fat was typically greater than histological measurement.

Concordance correlations were calculated using the 'concord' package in the statistical package STATA. Results show a modest concordance (rho c 0.781) although this does approach 1 (95% CI: 0.547, 1.014). This results both from a lack of perfect correlation (Pearson's r = .866) and from bias (C b = .902). The reduced major axis reveals a slope less than one (0.803 (thus, the true variance does not rise as rapidly as the incorrect values)) and a negative intercept (-0.439) (figure 3.6). The coefficient of variation was 0.63.
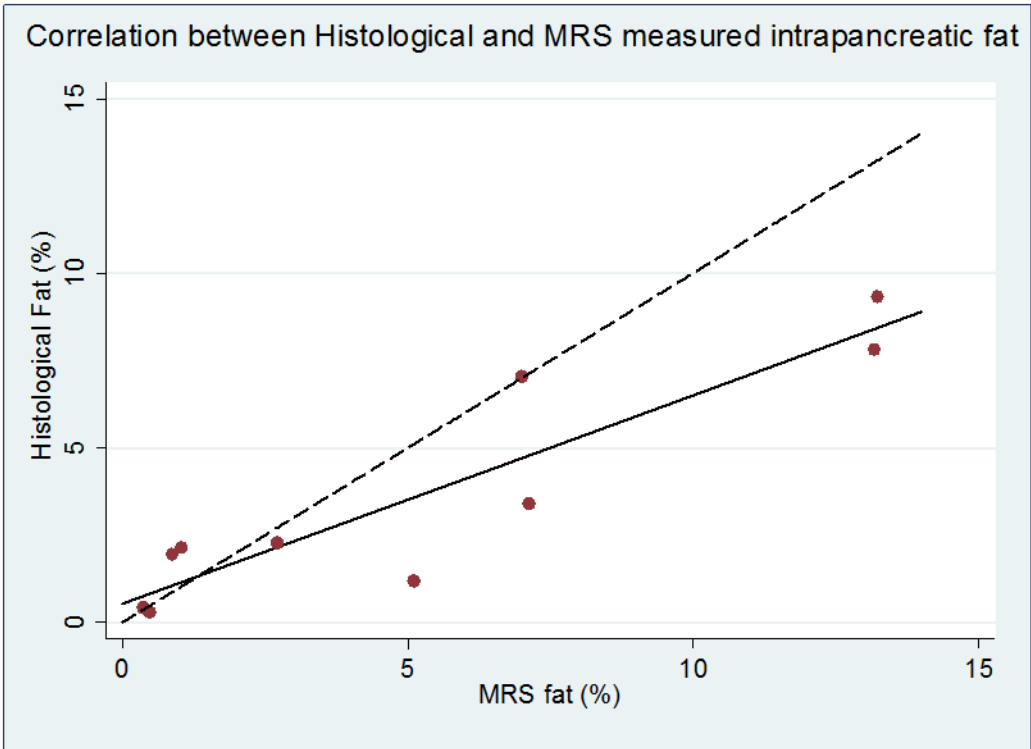
Figure 3.6: Correlation between histological and MRS measured intra-pancreatic fat. Solid line - line of best fit. Dashed line - line of perfect fit. MRS measurement of intra-pancreatic fat was typically greater than the histological measurement of intra-pancreatic fat.

3.5.2 PANCREATIC CS-MR

Visual assessment using Bland-Altman plots (figure 3.7) show moderate levels of agreement between CS-MR measurement and histological measurement of pancreatic fat.
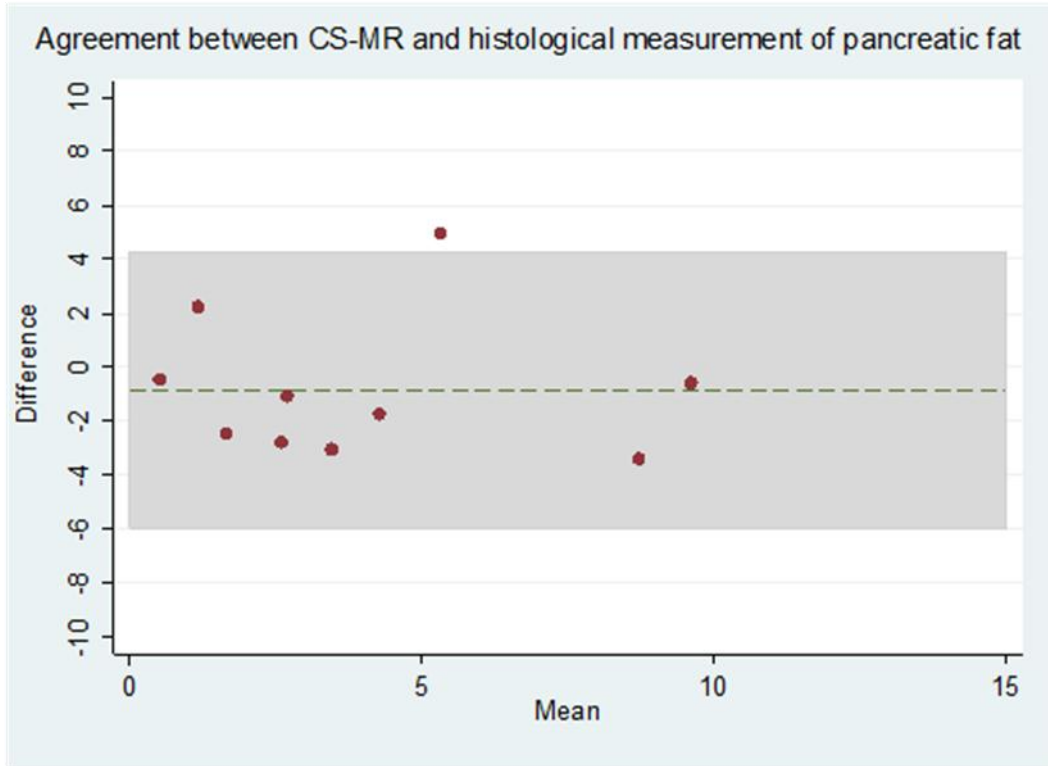
Figure 3.7: Bland Altman plot of agreement between CS-MR and histological measurement of pancreatic fat. The grey area represents 95% confidence interval and the green dashed line the average difference between measurements. This graph demonstrates that CS-MR measurement of pancreatic fat was typically greater than histological measurement.

Concordance correlations were calculated using the 'concord' package in the statistical package STATA. Results show a modest concordance (rho c 0.672). This results both from a lack of perfect correlation (Pearson's r = .696) and from bias (C b = .966). The reduced major axis reveals a slope of almost one (0.951 (thus, the true variance rises as rapidly as the incorrect values)) and a negative intercept (-0.616) (figure 3.8). The coefficient of variation was 1.92.
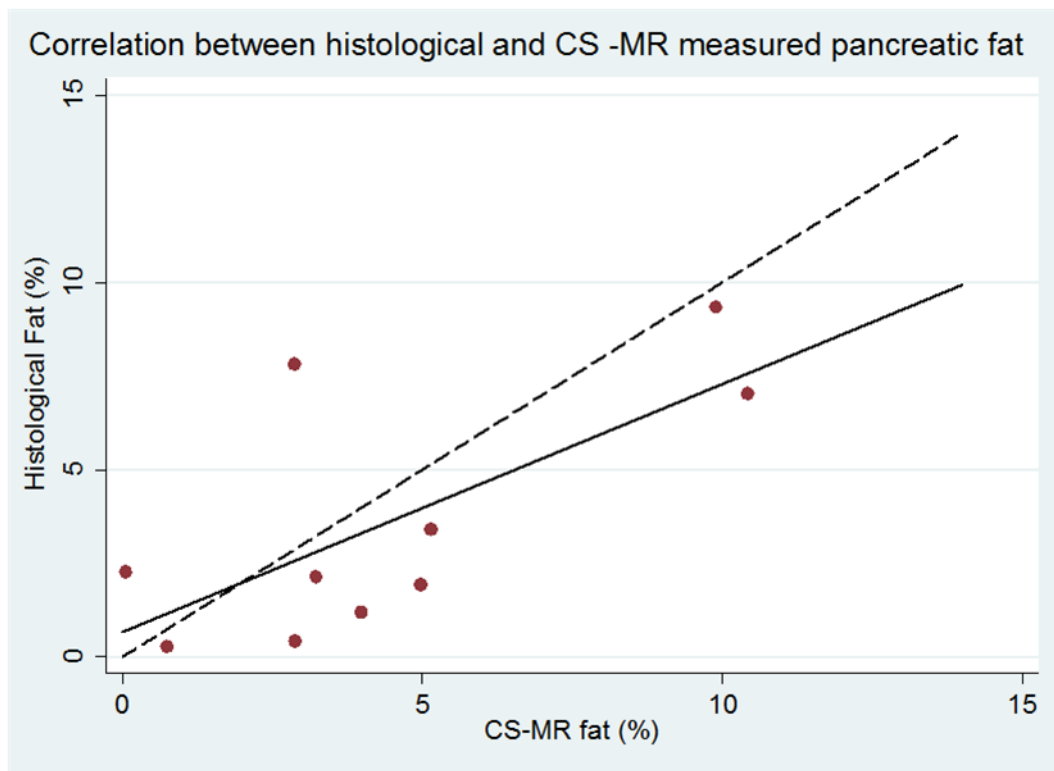
Figure 3.8. Correlation between histological and CS-MR measured intra-pancreatic fat. Solid line - line of best fit. Dashed line - line of perfect fit. CS-MR measurement of intra-pancreatic fat was typically greater than the histological measurement of intra-pancreatic fat.

3.6 COMMENTS ON INTRA-PANCREATIC VERSUS INTRA-HEPATIC FAT ASSESSMENT BY MR

Table 3.2 summarises the median and ranges for fat quantifications by MRS and CS-MR in the liver and pancreas in the 12 patients undergoing surgery with pancreatic pathology. The median values in the pancreas tend to be higher than those for the liver.

Additionally, within-person correlations between imaging acquired fat levels in the pancreas and liver were generally poor in these patients undergoing surgery with pancreatic pathology. This contrasts with data from healthy volunteers presented later in Chapter 4, which shows good within-person correlations between image-acquired fat levels in the pancreas and liver.

Thus, the setting of acquired tissue in patients undergoing pancreatic surgery with pathology was useful to test the accuracy of image-acquired fat levels in the pancreas with those on histological quantification, this setting has limitations in terms of the wider question in this thesis – the development of intra-pancreatic fat quantification using MRS and CS-MR as a risk susceptibility biomarker in healthy individuals.

3.7   MRS OF PANCREATIC AND BILIARY MASSES

In addition to calculating fat fraction within the pancreatic resection margin, this study was an opportunity to obtain MRS data from tumours of the pancreas pre-operatively. Discernible peaks in pancreatic ductal adenocarcinoma (figure 3.9) corresponded to water and lipid, in both a pancreatic pseudocyst (figure 3.10) and a pancreatic neuroendocrine tumour (figure 3.11) no fat was observed. However, similar MRS spectra, i.e. spectra without any fat were obtained from the normal pancreas in some cases.
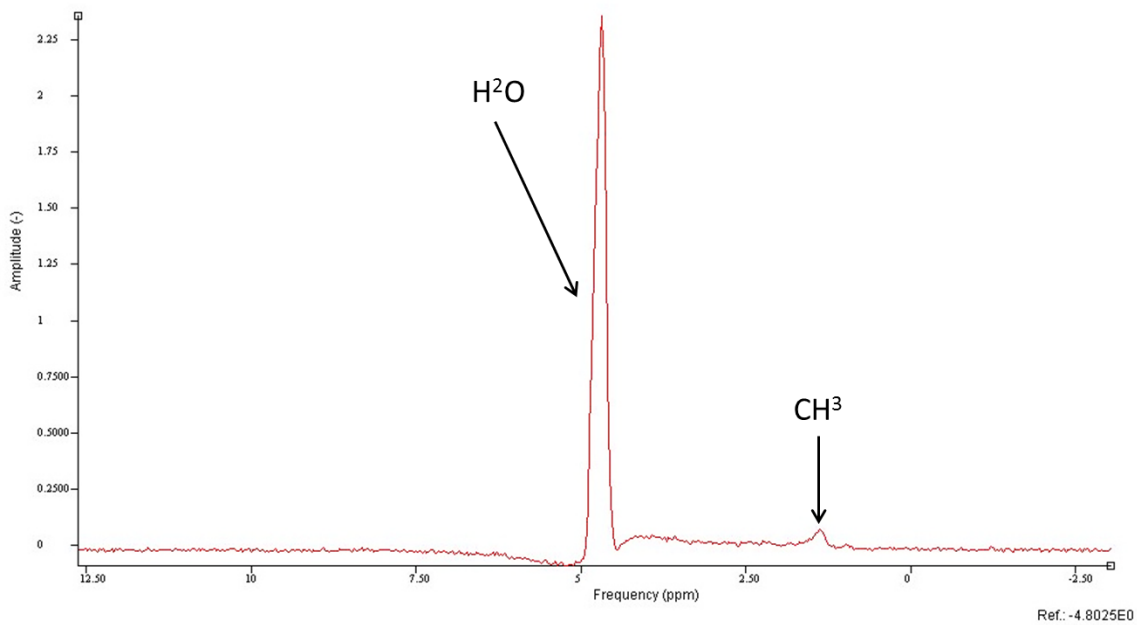
Figure 3.9: MRS data from large pancreatic tumour. Predominant water and CH3 (lipid) peaks are seen.
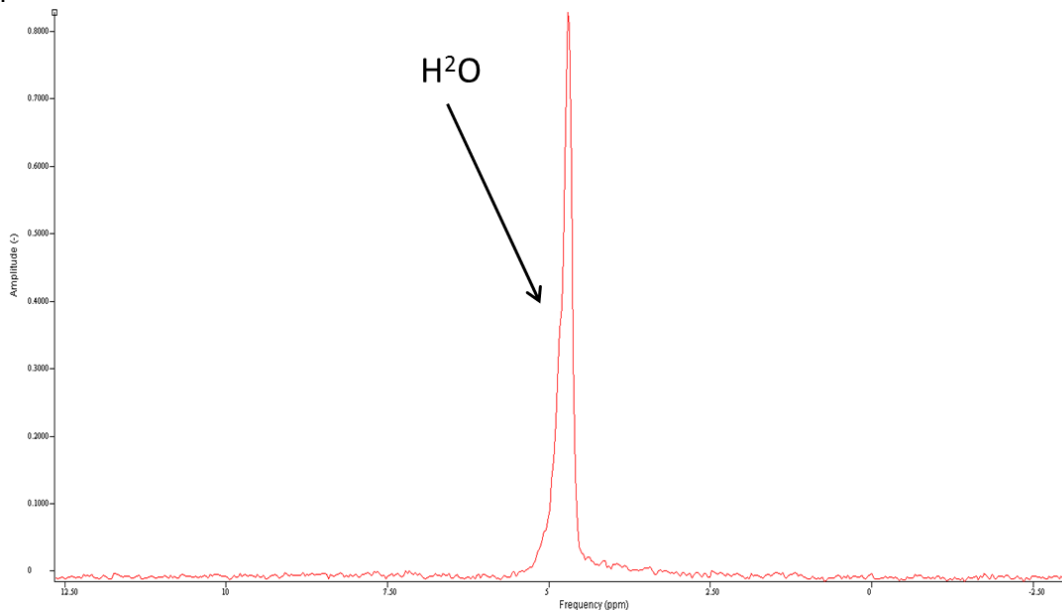
Figure 3.10: MRS data from pancreatic pseudocyst. Predominant water peak only is seen.
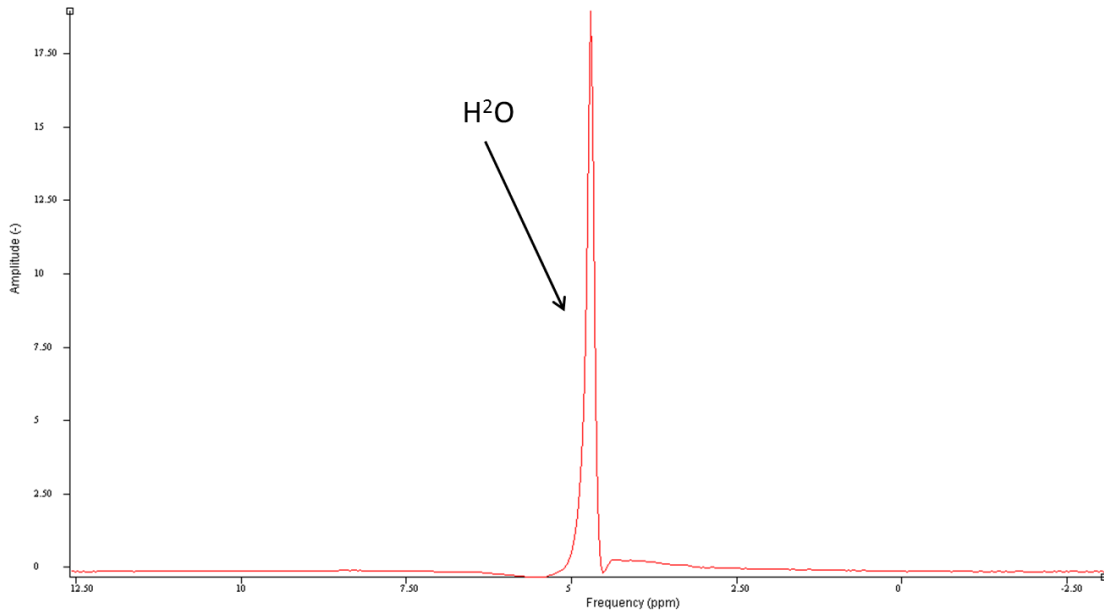
Figure 3.11: MRS data from pancreatic neuroendocrine tumour. Water peak only is seen.

3.8 DISCUSSION

3.8.1 MAIN FINDINGS

- CS-MR and MRS can be used to measure intra-pancreatic fat. These scans were well tolerated by pre-operative patients and these measurements have moderate/strong correlations with histological assessment of pancreatic fat (rho 0.781 and 0.672 respectively).

- The digital histological quantification is not in itself a perfect standard of pancreatic fat quantification (for example, in animal experience, the standard is biochemical fat quantification), but it is a key clinically relevant and accessible endpoint. Within these imperfections, the obtained concordance values are acceptable, and indicate that pancreatic assessment by MRS or CS-MR are fit for purpose.

- A question was considered – is MRS 'better' than CS-MR? Both MRS and CS-MR tend to overestimate the 'true' histological fat quantification, especially with increasing values of histological fat. The ranges of levels of agreement were moderately wide for both imaging modalities, but numbers were small and it was not possible to conclude that one was 'better' than the other.

- Using a 1.5T scanner, the discernible peaks on MRS for pancreatic ductal adenocarcinoma corresponded to the same lipid and water peaks that are seen in normal pancreatic tissue. There is therefore no role for 1.5T MRS in the evaluation of a pancreatic mass.

- In patients undergoing operations for pancreatic cancer, intra-pancreatic fat does not correlate with other markers of excess adiposity. This finding emphasises the need to use healthy participants in studies assessing the effect of intra-pancreatic fat on disease.

3.8.2 COMPARISON WITH THE LITERATURE

Although previous studies have compared different MR techniques or performed similar studies in animals, this is the first study to use the MR imaging techniques MRS and CS-MR to quantify pancreatic fat and to compare these results with histological quantification in humans

Lee et al used a relative signal intensity difference (RSID) technique to quantify pancreatic fat and compared these results with digital quantification of histology samples in a method that may be similar to this thesis but is poorly described within their paper.[149] Although the authors found a modest (rho 0.560) correlation between these measurements (figure 3.6) there are important differences between the paper and this thesis. Importantly, an RSID technique does not try to calculate the proportion of fat within an organ, instead this technique compares MR signal intensity with MR signal intensity of the spleen. This is illustrated by figure 3.8 taken from Lee et al. Here, the RSID x-axis runs from -30% to +30% demonstrating that this technique does not calculate a pancreatic fat proportion.
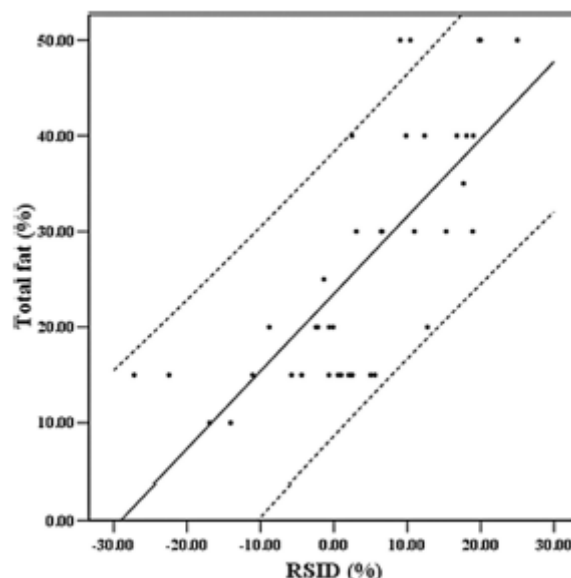
Figure 3.12 Lee et al's[149] scatter graph of relative signal intensity decrease on the x axis against histologically determined fat on the y axis. Rho is 0.560 but agreement is poor and the line of best fit does not pass near 0 on either axis.

Two authors[100, 107] have validated MRS by comparison with animal tissue with similar results to those seen here. Lingvay et al and Hannukainen et al compared MRS PFF with biochemical PFF using rats and pigs, respectively; Lingvay found an intra-class coefficient

(ICC) of 0.91 and Hannukainen an r^2 of 0.876 indicating good correlation.[100, 107] These results were important to demonstrate the feasibility of MRS to measure pancreatic fat. However, both these studies performed MR imaging on anaesthetised animals. This gives control over respiration and therefore voxel placement that is not possible in humans, which emphasises the need for the human validation seen in this thesis.

3.8.3 STRENGTHS AND WEAKNESSES

Our study attempting to determine if MRS and CS-MR are accurate MR imaging modalities to measure intra-pancreatic fat has several strengths: (i) We compared our MRS and MR measurements to a method of histological validation shown by our group to have good inter-rater and intra-rater variability.[181] (ii) The MRS and CS-MR methods used here have good inter-rater variability. (iii) We used MR methods that were tolerated by all patients in this study so are likely to be applicable to the general population. (iv) The range of pancreatic fat measured here is consistent with the reported range of pancreatic fat in the healthy population.[100, 231]

There are weaknesses to this study: (i) the MR measurement and histological measurements of pancreatic fat are different. Histological assessment of pancreatic fat is by digital determination of fat percentage which assesses the proportion of a histology slide occupied by fat. Additionally both MRS and histology measurements only sample the fat in local regions which can introduce variability. Comparatively, CS-MR and MRS calculate a percentage fat by weight. (ii) As this study was performed in patients with pancreatic diseases the majority of patients had pancreatic fat less than 5%. This may be important if the degree of pancreatic fat infiltration that is important to disease is the same as for the liver, above 5%.[232]

3.8.4 SUMMARY

Our finding that MRS and CS-MR overestimated intra-pancreatic fat may be an important clinical one. Currently, no conclusive evidence for the clinical implications for intra-pancreatic fat are known[147] and therefore, there are no data on the level of intra-pancreatic fat that can be considered harmful. However, should data emerge it will be important to remember that different methods of assessing intra-pancreatic fat may produce different absolute levels.

4 PRECISION OF MRS AND CS-MR FOR PANCREATIC FAT QUANTIFICATION

> **Precision, or reproducibility, is the extent to which repeated measurements vary under the same conditions.**

The development of imaging biomarkers has to undergo a process, from discovery, through verification, validation and qualification before they can be used in clinical routine.[230] Stage-one of the PanORAMA project dealt directly with one aspect of biomarker method validation, accuracy. In stage-two of the PanORAMA study we now turn to another aspect of method validation, precision. Precision is the degree to which repeated measurements under the same conditions show the same results and is also termed reproducibility. As discussed in the opening of chapter 3, prior to embarking on assessment of a biomarker acceptable levels of precision should be set. For assay analysis, samples are expected to vary by less than 15%, except at the lower limit of quantification, where 20% is allowable.[227] For imaging biomarkers, levels of precision are yet to be determined.[230]

To maximise outputs the study was designed not just to address precision but to move to the next step of biomarker evaluation, qualification. Qualification is the evidentiary and statistical process linking a biomarker to biologic and clinical endpoints. My literature review demonstrates evidence of a link between excess adiposity, measured as body mass index (BMI), and pancreatic cancer. I therefore sought to investigate the relationship between BMI and intra-pancreatic fat, as my hypothesis is that this ectopic fat store is a better marker for excess-adiposity driven pancreatic cancer. Stage-one of the PanORAMA study demonstrates a lack of relationship between adiposity measurements and intra-pancreatic fat. However, the study was performed in patients with known pancreatic disease, which might have confounded results. As a potential biomarker for pancreatic cancer development it is important that relationships are assessed in healthy patients without pancreatic disease (the focus here). Stage 2.1 of the PanORAMA study was therefore performed in healthy volunteers with the aim to assess the relationships between intra-pancreatic fat, anthropometric markers of excess adiposity and systemic and ectopic fat stores.[233]

Initial results of stage 2.1 demonstrated poor reproducibility for pancreatic MRS. Following this, I made adjustments to our MRS protocol and sought additional funding to test these changes. The stepwise development of these protocol changes are described here and the additional study was funded by an MR development grant from the University of Manchester (stage 2.2).

4.1 STAGE 2.1 PANORAMA METHODOLOGY SUMMARY

The technical methodology used to acquire CS-MR and MRS data from the pancreas is covered fully in chapter 2. For this study, data from three MRS voxels placed within the pancreatic head, body and tail and three CS-MR regions of interest (ROIs) from the same locations (figure 4.1) were obtained in a group of volunteers without known pancreatic disease. To test reproducibility, volunteers underwent repeat scans in the same sitting, after initial image acquisition, volunteers came off the MR imaging table and an entirely new sequence performed. Additional image sequences to calculate intra-hepatic, visceral and subcutaneous fat and other anthropometric measures of excess adiposity were collected during the first sitting.
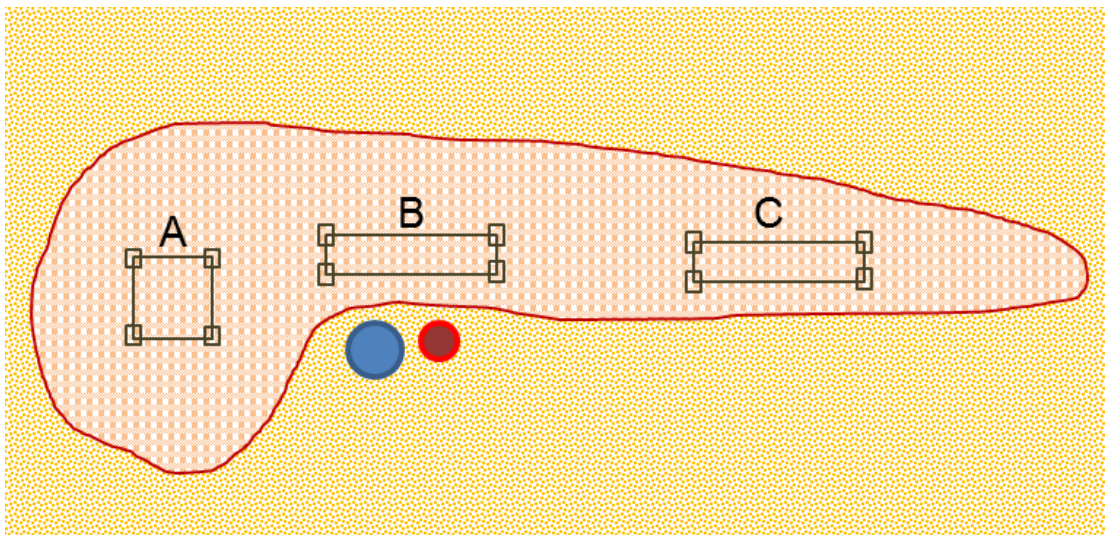
Figure 4.1: Pancreatic schematic demonstrating positioning for voxels (black rectangles) in MRS or ROIs for CS-MR data acquisition in stage 2. Data were acquired from the pancreatic head (A) body (B) and tail (C).

4.2   STAGE 2.1 PANORAMA VOLUNTEER CHARACTERISTICS

Fifteen volunteers were recruited (seven male, eight female). All underwent MR imaging and measurement of anthropometric indices (waist circumference, waist-hip ratio, BMI). The characteristics of these volunteers are summarised in table 4.1. The recruited volunteers had a greater range of ectopic fat than those in chapter 3, presumably as they were healthy volunteers.

Table 4.1: Summary characteristics of Stage 2.1 PanORAMA volunteers

	Median (range) n=15
Age (years)	45 (27 – 58)
BMI (kg/m^2)	29.3 (20.2 – 37.78)
Waist circumference (cm)	100 (73 – 126)
Waist to hip ratio	0.92 (0.72 – 1.22)
MRS measured mean intrapancreatic fat (%)	6.3 (0.04 – 17.8)
CS-MR measured mean intrapancreatic fat (%)	5.4 (1.6 – 15.8)
MRS measured intrahepatic fat (%)	2.9 (0.36 – 22.0)
Visceral fat volume (cm^3)	2203 (288 – 5922)
Subcutaneous fat volume (cm^3)	3888 (1412 – 12614)

4.3   PANCREATIC MRS SPECTRA IN HEALTHY VOLUNTEERS

MRS spectra from the pancreas were obtained from all participants. Discernible metabolites varied with the proportion of pancreatic fat. Spectral peaks were obtained for water and the predominant CH_2 (methylene) bond present in lipids (figures 4.2 and 4.3). Only at percentages of fat above 15%, other fat peaks become apparent (figure 4.4).
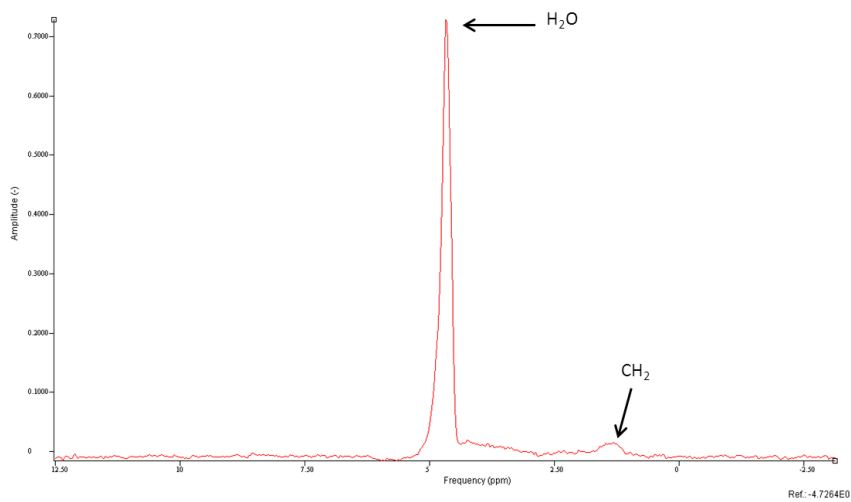
Figure 4.2: Volunteer with 3.5% pancreatic fat.
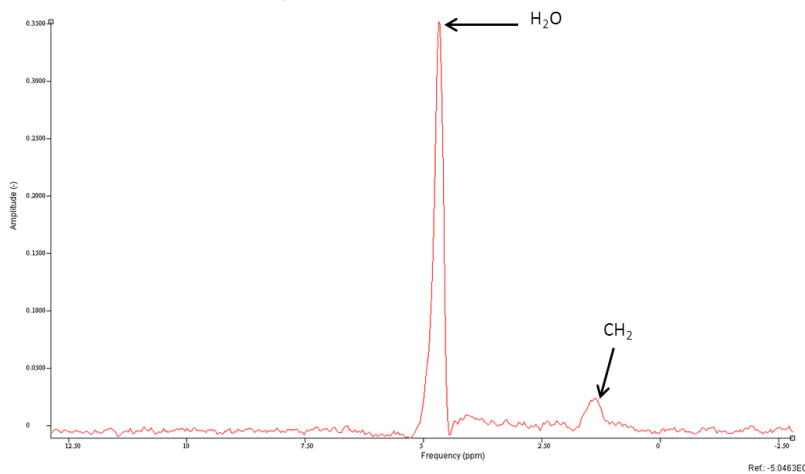
Figure 4.3: Volunteer with 11% pancreatic fat.
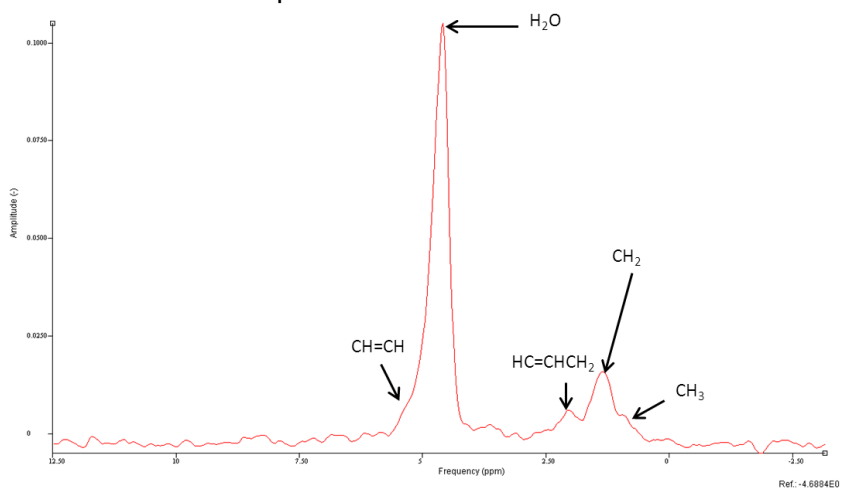
Figure 4.4: Volunteer with 17% pancreatic fat. Discernible peaks correspond to methylene (CH2), methyl (CH3), α-carboxyl and α-olefinic (HC=CHCH2), methane (CH=CH).

4.4 VISUAL ASSESSMENT OF PANCREATIC FAT DISTRIBUTION IN HEALTHY INDIVIDUALS

The acquisition of MRS data and CS-MR images requires planning images. These images, T2 weighted to highlight adipose tissue, demonstrated variation in the visceral fat-pancreas interface. Two phenotypes were observed: For most volunteers this interface was clearly defined and the distinction between pancreatic tissue and surrounding visceral fat clear (type I, figure 4.5A). For some volunteers, the pancreatic border is disrupted by adipose tissue and 'islands' of adipose tissue were visible within the pancreas (type II, figure 4.5B). Potentially, for type II pancreata there is inhomogeneity in fat distribution, this would have implications for studies wishing to measure intra-pancreatic adipose tissue and requires further evaluation.
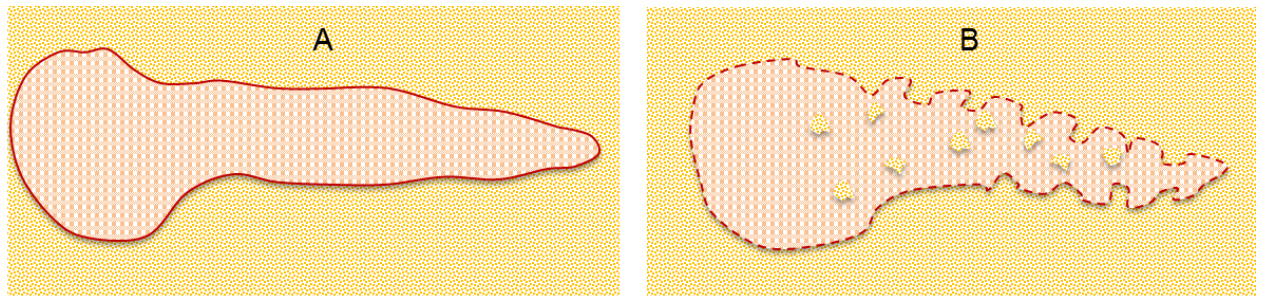
Figure 4.5: Schematic demonstrating different pancreatic phenotypes with respect to adipose tissue. Proposed type I (A) has a smooth visceral-fat pancreas border, in type II (B) the interface between the pancreas and surrounding fat is disrupted and islands of adipose tissue are visible within the pancreas itself.

A:
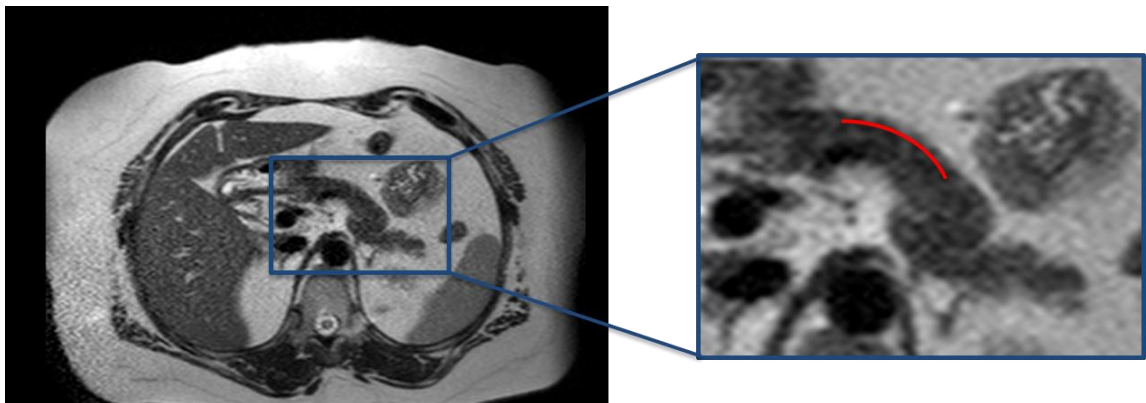
B:
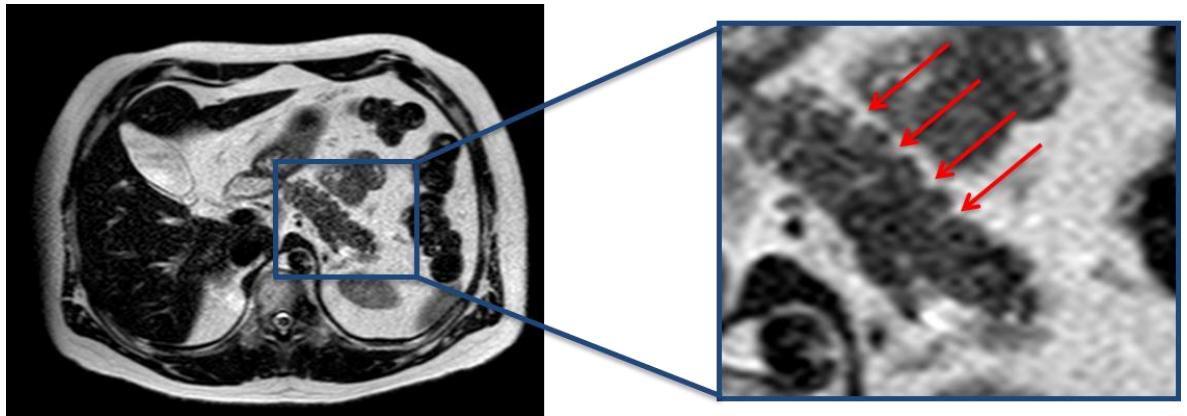
C:
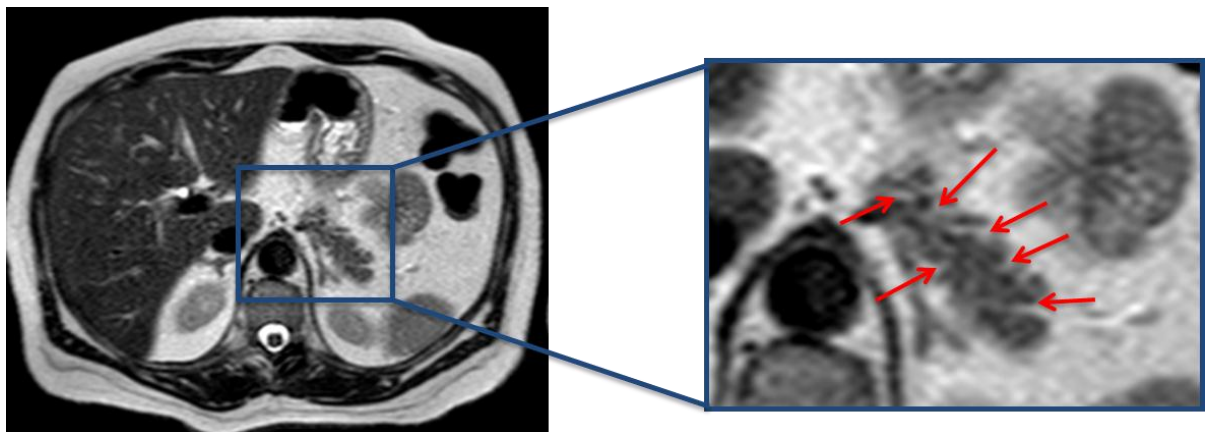
Figure 4.6: Axial magnetic resonance images demonstrating variation in the pancreatic-visceral fat interface. (A) The interface is smooth (red line) and with a clear boundary between pancreas and surrounding adipose tissue. (B) The interface is marked by indents of adipose tissue that run up to half way along the pancreas (red arrows). The pancreas itself has mixed signal intensity. (C) There is a lack of a distinct interface and the pancreatic tissue appears to blend into the surrounding adipose tissue.

4.5   CS-MR AND MRS MEASUREMENT OF INTRA-PANCREATIC FAT: PRELIMINARY PROTOCOL

All volunteers underwent same-day repeated measurements of CS-MR and MRS pancreatic fat. Bland Altman plots show good levels of agreement for CS-MR (figure 4.7) i.e. the agreement line was close to zero; the upper and lower limits of agreement were reasonably narrow; and the agreement as similar across ranges. This was not the case for MRS (figure 4.8). Bland Altman plots for repeated measurement of mean pancreatic fat demonstrate similar acceptable agreement for CS-MR but not for MRS (figure 4.9). The coefficient of variation (CV) for repeated measurement of pancreatic head, body and tail fat were 64%, 77%, 91% for MRS and 33%, 31%, 36% for CS-MR respectively. For repeated mean pancreatic fat (i.e. mean across all sites), CV was within acceptable limits for CS-MR (20%) but not MRS (64%).
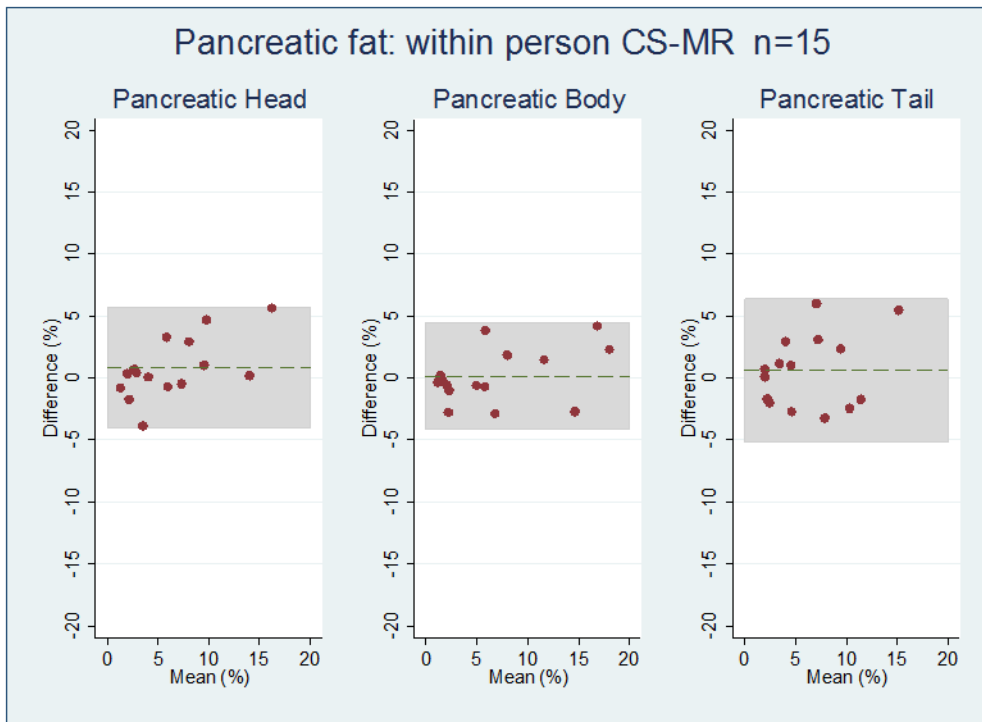
Figure 4.7: Bland Altman plot of agreement between repeated measurements of pancreatic fat using CS-MR. These graphs demonstrate that CS-MR measurement of pancreatic fat had a variability of up to 5% for pancreatic fat up to 20%. Reproducibility was best for the pancreatic head and body.
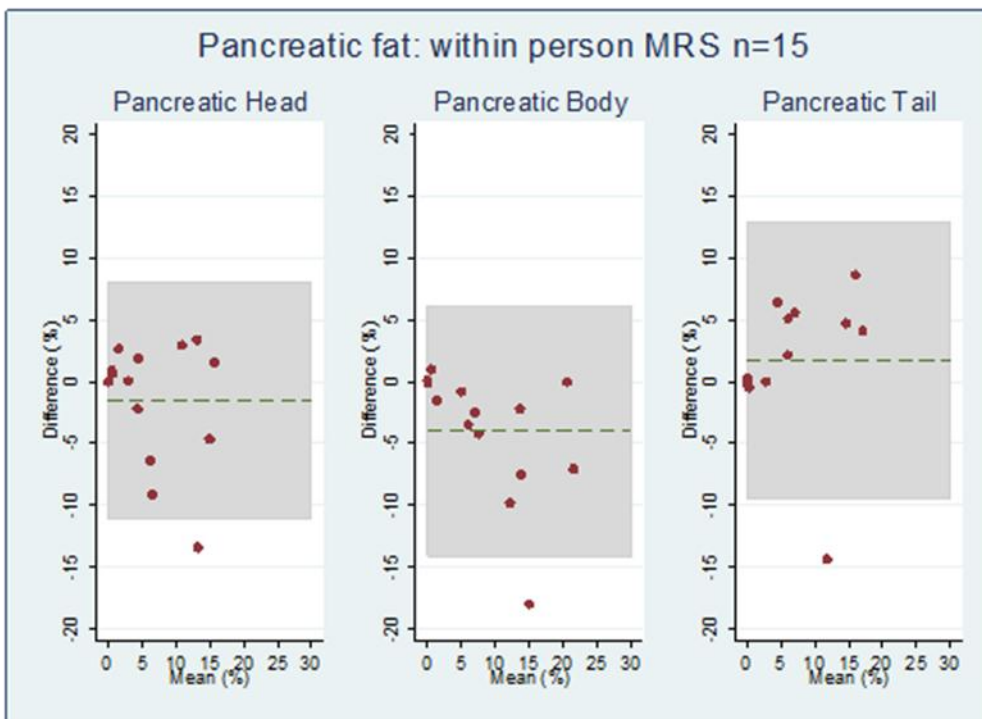
Figure 4.8: Bland Altman plot of agreement between repeated measurements of pancreatic fat using MRS. These graphs demonstrate that MRS measurement of pancreatic fat had poor reproducibility.
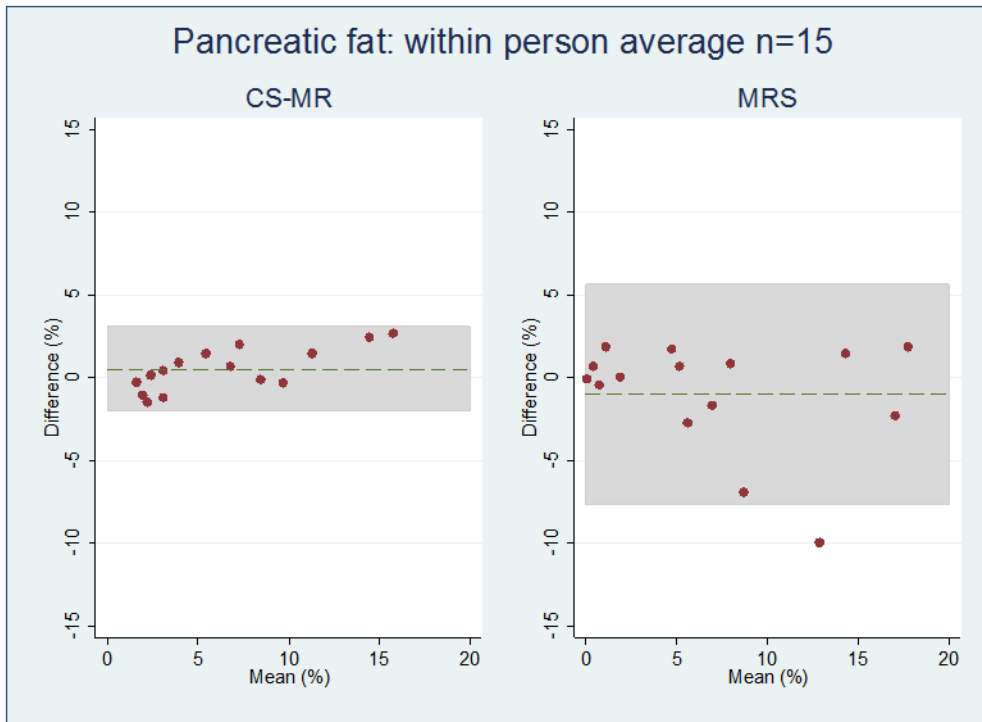
Figure 4.9: Bland Altman plot of agreement between average of repeated measurements of pancreatic fat using MRS and CS-MR. For CS-MR (left) the 95% confidence interval is less than 4% for intra-pancreatic fat up to 20%. For MRS (right) there remained poor reproducibility despite the use of average values.

4.6 RELATIONSHIPS WITH OTHER ANTHROPOMETRICS

CS-MR values for pancreatic fat were correlated with other anthropometric measurements of excess adiposity. Moderate correlations (Table 4.2) were found with other measurements demonstrating variability in body fat distribution between individuals. MRS values for pancreatic fat are not reported here as we had concerns about the reproducibility of these data.

Table 4.2: Correlations between measures of excess adiposity in Stage 2.1 PanORAMA volunteers. Values shown are for Spearman rho. Significant correlations were seen between many measurements of excess adiposity. The strength of these correlations were often only moderate (CS-MR Pancreatic fat and WC, rho 0.56). This supports our hypothesis of variation in ectopic fat deposition.

	CS-MR Pancreatic fat (%)	MRS hepatic fat (%)	CS-MR hepatic fat (%)	BMI (kg/m^2)	WC (cm)	WHR	Age (years)	Visceral adipose tissue (cm^3)
MRS hepatic fat (%)	0.3107							
CS-MR hepatic fat (%)	0.4071	0.9571						
BMI (kg/m^3)	0.5571	0.6821	0.6571					
WC (cm)	0.5648	0.8472	0.7954	0.8811				
WHR	0.5487	0.8150	0.8132	0.7024	0.9186			
Age (years)	0.5510	0.1503	0.1878	0.2898	0.1979	0.1352		
Visceral adipose tissue (cm^3)	0.6429	0.6357	0.6321	0.7107	0.8293	0.8651	0.3685	
Subcutaneous adipose tissue (cm^3)	0.5393	0.5536	0.5536	0.8714	0.7239	0.5469	0.1843	0.5786

Significant relationships (p=<0.05) in blue.

4.7   PANORAMA EXTENSION PROJECT STAGE 2.2

Stage 2.1 of the PanORAMA project revealed unacceptable variability for repeated measurements of pancreatic MRS. A number of potential improvements to our pancreatic MRS protocol were identified and a successful application for development scanning time was made to the University of Manchester for a Magnetic Resonance Imaging Facilities (MRIF) grant. This allowed us to recruit a further ten healthy volunteers without known pancreatic disease to undergo repeated (same-day) measurements of pancreatic head and body MRS and liver MRS.

4.7.1   PANCREATIC MRS: IMPROVED PROTOCOL

We considered that a likely source of variability in our reproducibility measurements was movement of the pancreas due to diaphragm movement during respiration. An MRS voxel is placed based on planning images. If the MRS data acquisition occurs in a different phase of respiration to the planning sequence then data may be acquired from a different anatomical location than intended. Typically for the pancreas this is the surrounding visceral fat. Five changes to the pancreatic MRS protocol were therefore made to test if we could improve reproducibility:

1. Reduced voxel depth (to 5mm x 40mm x 5mm) to reduce potential overlap into surrounding visceral fat.
2. Control of the phase of respiration in which planning sequences are taken and MRS data acquired. This was achieved using breath holds for planning sequences and respiratory triggered MRS sequences.
3. The need for an additional oblique pancreas planning sequence to improve pancreas voxel placement (figure 4.10).
4. Dynamic collection MRS data. Sixteen MRS spectra are acquired and averaged for every STEAM sequence. To ensure the quality of this data we collected each spectra individually and inspected them in an additional post-processing step.
5. Pancreatic voxel placement was limited to the head and body as the tail had shown particularly high variability possibly due the fact it tapers as it nears the spleen.
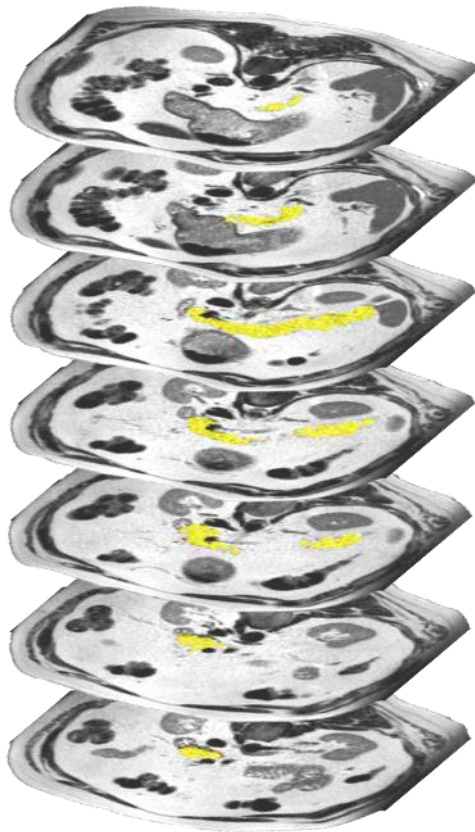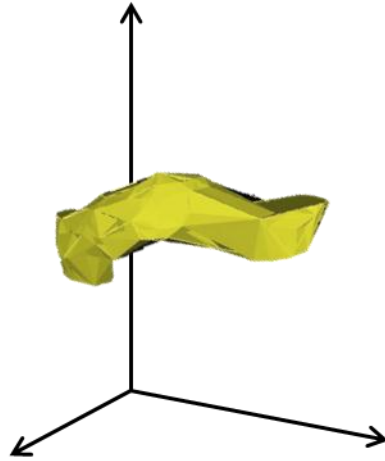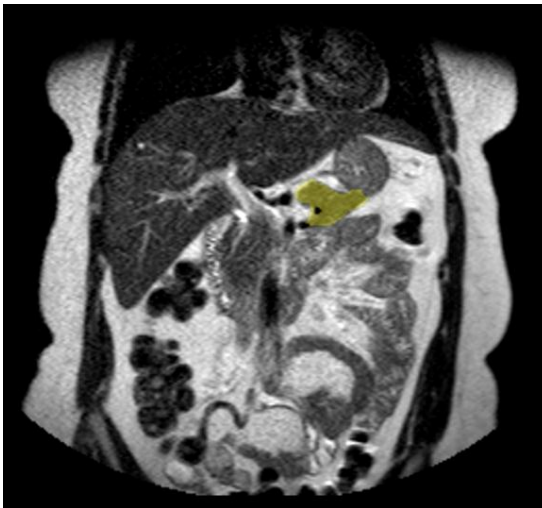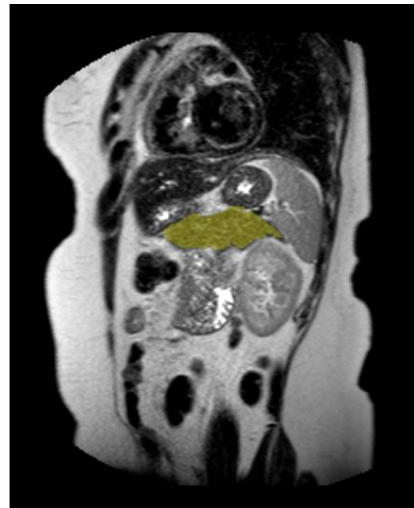
Figure 4.10: Axial abdominal MR images with pancreatic tissue highlighted in yellow (A). 3-D reconstruction of the pancreas (B). Note that the body of the pancreas runs obliquely in both the cranio-caudal and anterior-posterior axis. The long axis of the pancreatic body-tail therefore lies obliquely and is poorly visualised by coronal imaging (C). Oblique images along this axis allow better visualisation of the pancreatic body for MRS voxel placement (D).

4.8   Precision of Pancreatic MRS after new protocol

Moderate levels of agreement were observed between repeated measurements of pancreatic head and pancreatic body fat using MRS (Figures 4.11 and 4.12). The coefficient of variation for these repeated measurements was 25% (pancreatic head) and 28% (pancreatic body). Intra-class correlation (rho c = 0.686) and Spearman's correlation (rho = 0.772) showed moderate levels of agreement for repeated measurements of pancreatic head fat. For repeated measurements of pancreatic body fat, intra-class correlation (ICC rho c= 0.857) and Spearman's correlation (rho = 0.811) were good and the coefficient of variation acceptable at 15%.
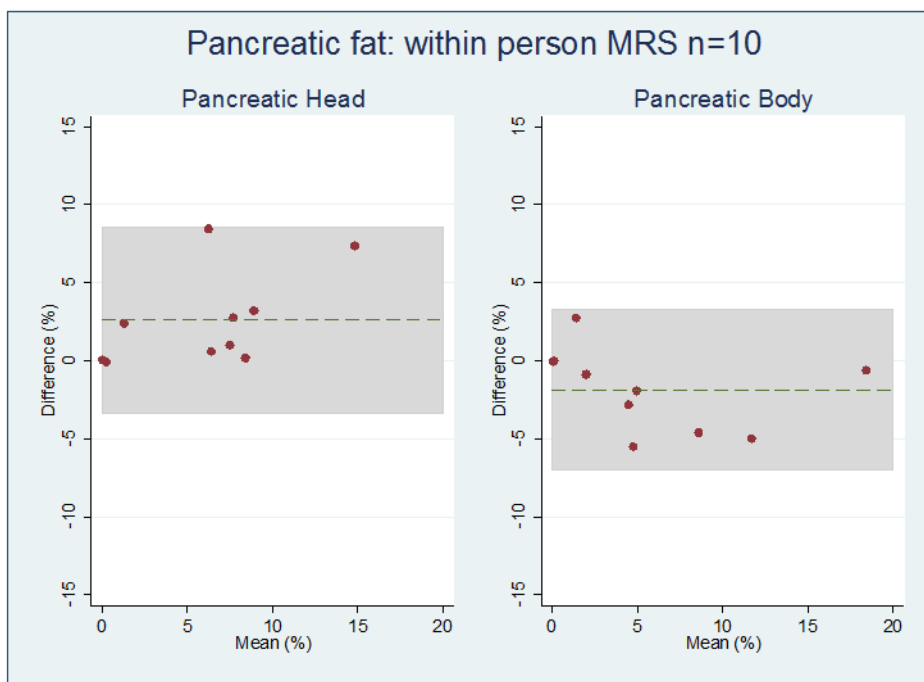
Figure 4.11: Bland Altman plots of repeated MRS measurements of pancreatic head and body fat. The grey shaded area demonstrates that the majority of values remain within 5% for both repeated measurement of pancreatic head and body fat.
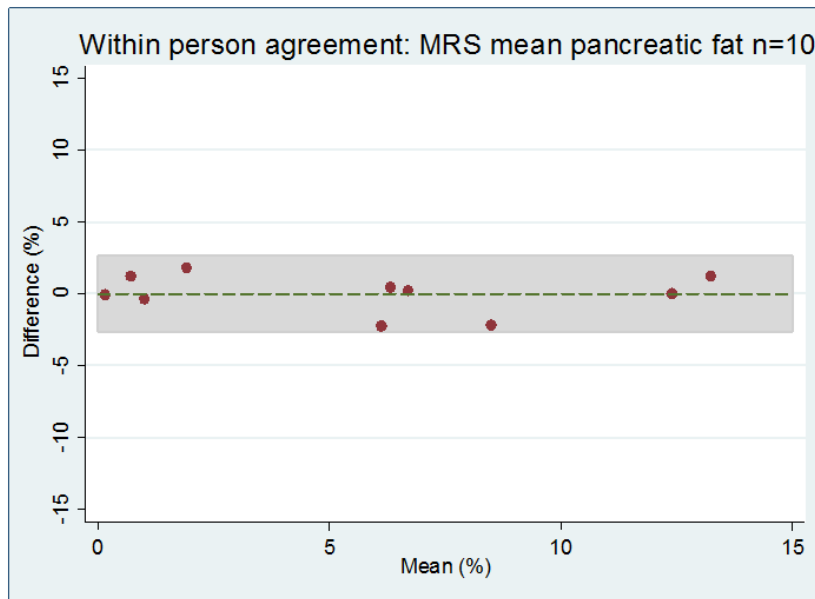
Figure 4.12: Bland Altman plots of repeated MRS measurements of mean pancreatic fat. Reproducibility was acceptable as the majority of values remain within 2.5% for both repeated measurement of pancreatic fat.

4.9 PRECISION OF HEPATIC MRS

Good levels of agreement were observed between repeated measurements of hepatic MRS (Figure 4.13) with no trend to loss of agreement at increasing hepatic fat levels and a coefficient of variation of 15%. Acceptable levels of intra-class correlation (rho c = 0.974) and Pearson's correlation (0.995) were found.
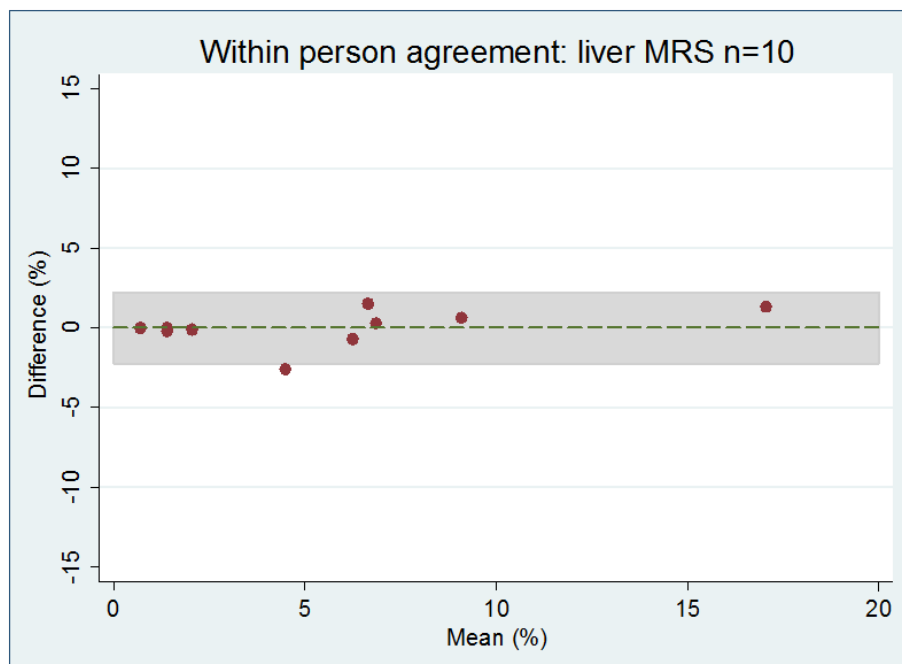
Figure 4.13: Bland Altman plots of repeated MRS measurements of hepatic fat. Here I demonstrate good reproducibility for repeated measurement of hepatic fat across a range of values of intra-hepatic fat.

4.10 DISCUSSION

4.10.1 MAIN FINDINGS

- CS-MR and MRS can be considered to be fit-for purpose as a potential methods of biomarker quantification.

- Our initial study demonstrated that CS-MR has acceptable precision to measure intra-pancreatic fat. The precision is improved by multiple measurements and was superior to MRS.

- After refinement of MRS protocols, MRS has acceptable precision. The precision is improved by multiple measurements. MRS has an advantage in that it allows the identification of multiple lipid peaks at high pancreatic fat proportions.

- We have confirmed findings from previous studies that demonstrate that Hepatic MRS has acceptable precision for single measurements.

- Intra-pancreatic fat correlates with established anthropometric markers of excess adiposity; BMI, WC, WHR. This is an important finding as increasing BMI, WC and WHR are linked with increased risk of pancreatic cancer. Yet, correlation with these markers is only moderate. This finding supports the need for further research that considers organ specific calculation of excess-adiposity and the relationship with cancer-risk. For the development of pancreatic cancer, research is warranted into whether intra-pancreatic fat is a better marker of the risk attributable to excess adiposity.

- Visually, I observed variation in the pancreas-visceral fat interface and variation in signal intensity within the pancreas. This may contribute to variability in intra-pancreatic fat measurement.

4.10.2 COMPARISON WITH THE LITERATURE

Previous authors investigating associations between intra-pancreatic fat and other markers of excess adiposity have found inconsistent results. Most studies have found an association between BMI with PFF. Lingvay et al found a 7-fold increase in PFF in patients with a BMI 32.4 ± 6.1 kg/m^2 compared with BMI of 22.2 ± 1.6 kg/m^2.[100] Similarly, Maggio et al found $4.8 \pm 1.9\%$ and $3.6 \pm 0.9\%$ PFF in obese (BMI 30.3 ± 5.4 kg/m^2) and lean (18.9 ± 1.9 kg/m^2) adolescents respectively.[101] In contrast, Patel et al in a cohort of patients with non-alcoholic fatty liver disease and Lee et al in 293 overweight or obese patients found no association between BMI and PFF.[102, 103]

An association between increased VAT and PFF has been reported by some,[103-105] but not all.[106, 107] Rossi et al found VAT to be the main predictor of PFF in a study assessing body fat distribution, inflammatory markers, adipocytokines in obese men and women.[105] Heni et al found a significant correlation between VAT and PFF in a model adjusted for age and gender.[104] Lee et al assessed pancreatic fat using USS in a multivariable model and found VAT to be the determinant factor in PFF over BMI.[103] Conversely, both Van der Zijl in a study of age and BMI matched individuals and Hannukainen in a study of monozygotic twins found no associated between VAT and PFF as measured by MRS.[106, 107]

The reasons for the differences between these studies are this are unclear but are likely to be due to heterogeneity of inclusion criteria, methods of quantification and small study groups. Overall, those studies that attempted to quantify intra-pancreatic fat found similar ranges of intra-pancreatic fat to this study. Both Lingvay et al and Hannukainen et al found a range of between 0% to 18% PFF in adults. Hanunkainen found positive associations on regression analysis with intra-hepatic fat and SAT.[100, 107]

This is the first study to use Bland Altman methods to assess the precision of MRS and CS-MR to measure intra-pancreatic fat. Only one previous study has reported precision, Lingvay et al have similarly reported good reproducibility of MRS (ICC 0.94) by repeating their investigation in a subset of volunteers at 2 weeks.

4.10.3 STRENGTHS AND WEAKNESSES

Our study to determine if MRS and CS-MR are precise MR imaging modalities to measure intra-pancreatic fat has several strengths. Firstly, the study was performed in the population of interest i.e. healthy volunteers. Secondly, the CS-MR and MRS methods were well tolerated. Thirdly, the range of pancreatic fat measured here is consistent with the reported range of pancreatic fat in the healthy population. Finally, unlike previous studies. our statistical methodology is consistent with biomarker assay guidelines.

However, there are weaknesses to this study. Firstly, my preliminary assessment of MRS as a tool to measure intra-pancreatic fat demonstrated unacceptable variation. However, I addressed this with a second study and demonstrated acceptable variation for repeated measurements of mean pancreatic fat. Secondly, as a correlation study, the number of volunteers is only small. Hence our findings are exploratory and hypothesis generating rather than conclusive.

5   BREAST RISK REDUCTION INTERMITTENT DIETARY EVALUATION 2 (BRRIDE-2)

The third stage of this research program was to test the hypothesis that the quantity of intra-pancreatic fat could be reduced with through dietary intervention i.e. it is modifiable. Here, we have moved beyond the initial stages of biomarker verification and validation to biomarker qualification (CTAAC/BIDD BM Qualification Stage 1). My original intention had been to test this hypothesis by collaborating with an already running dietary intervention trial, recruiting participants to undergo MR measurement of intra-pancreatic fat at the start and end of dietary intervention. Instead, during discussions with my collaborators, there was interest in utilising our experience with MR quantification of ectopic fat to develop an improved design of a randomised controlled trial to compare the effect of different dietary interventions on ectopic fat stores.

For my collaborators (Harvie, Higham, Howell), reduction in intra-hepatic fat was the primary outcome measure. This team are interested in obesity and post-menopausal breast cancer risk. Insulin resistance is a proposed mechanism for this increased risk with intra-hepatic fat considered a key driver of insulin resistance.[186, 187] The study was therefore designed with intra-hepatic fat reduction as the primary outcome measure. Reductions in intra-pancreatic fat are explorative due to the paucity of previous studies addressing this question so intra-pancreatic fat reduction was a secondary outcome within this study.

The BRRIDE-2 study (Breast Risk Reduction Intermittent Dietary Evaluation 2), a randomised controlled clinical trial comparing the effect of intermittent energy restriction with daily energy restriction on hepatic and pancreatic adipose stores and insulin resistance, ran between January 2015 and October 2015. There were three broad secondary outcome measures from this trial – changes in anthropometric measures; changes in advanced imaging; and changes in dynamic insulin resistance testing. The latter compromise a large set of analyses beyond this thesis. This study was part funded by Pancreatic Cancer UK, Help Against Liver Tumours (HALT) and Genesis Breast Cancer Prevention. The sponsor of this study was the University Hospital South Manchester and ethical approval granted by the Health Research Authority, National Research Ethics Service Committee South Central, Oxford B (14/SC/1097). The trial registry number was ISRCTN10803394 and the trial registered on the Cancer Research Network database (UKCRN ID 18052).

5.1 BRRIDE-2 METHODOLOGY SUMMARY

The methodology for the BRRIDE-2 study is outlined in chapter 2. To summarise, I did a randomised controlled trial comparing the effects a 25% energy restricted diet prescribed as either intermittent energy restriction (IER) or daily energy restriction (DER) on MR measured ectopic fat, anthropometric measures and metabolic markers of insulin resistance. Obese pre-menopausal women were randomised to one of the two 25% energy restricted diets, these provide 75% of their estimated energy requirements for an eight-week weight loss period. Both diets provided 30% energy from fat (15% MUFA, 8% PUFA, 7% saturated) 25% energy from protein and 45% from low glycaemic load carbohydrate and allows up to 10 units of alcohol per week:

- Intermittent energy restriction

A low carbohydrate energy restricted diet (600 kcal, <50g carbohydrate, 50 g protein day) (70% energy restriction) for two consecutive days and a modest energy restricted Mediterranean diet (7% energy restriction) for the remaining five days of the week.

- Daily energy restriction

A daily 25% energy restricted Mediterranean diet for seven days per week.

This was a follow up study of a previous randomised trial of IER versus DER.[188] which showed improvements in insulin resistance determined by HOMA-IR with both interventions over 6 months, but greatest improvements with IER despite comparable weight loss. As this trial did not include imaging quantification of ectopic fat it generated the hypothesis that IER may have a greater effect of intra-hepatic fat than DER. The greater improvements in HOMA in this trial were seen on non-restricted as well as restricted days. I hypothesised that the observed reductions in HOMA-IR may be due to a nadir in intra-hepatic fat associated with the 2 days of marked (70%) energy restriction.

Therefore, in the BRRIDE-2 study, all MR measurements and metabolic tests were repeated in week seven and at the end of the study. For participants in the IER group, MR assessment in week 7 immediately followed their 2 days of 70% energy restriction with week 8 assessment following at least 48 hours of normal energy intake. The specific aim was to test if the week-seven energy restriction was associated with reduced intra-hepatic fat in the IER group when compared with non-fasting in week 8. Anthropometric measurements (WC, WHR, weight and height) were taken when attending University Hospital South Manchester, at baseline, week 2, 4 and 8 (Figure 5.1).

	Week	
Patients informed of trial at breast clinic appointment or mail invite.		
Interested potential participant contacts team at UHSM, further discussion, screened for eligibility.		Recruitment
Potential participant contacted by lead researcher. Date of 'Visit 1' arranged. Asked to record 7 days normal diet (7 day food diary or web based my food 24 programme) and physical activity using IPAQ.		
Visit 1- WMIC and The Christies NHS Trust: Informed consent by lead researcher, MR imaging, OGTT, biochemical tests	0	Baseline measurements and randomisation
Visit - UHSM: Within same week of visit 1: Baseline measures of resting energy expenditure (REE), body fat, fat free mass (bioelectrical impedence), anthropometrics (weight, waist, hip and bust circumference), BP and pulse. Randomised to IER/ DER and given detailed dietary advice		
Intermittent Energy Restriction (n=14) — Daily Energy Restriction (n=14)		
Dietary intervention commences. All participants asked to record for the next 8 weeks daily dietary intake using written food diaries or web based my food 24 programme	1	
Telephone review with dietitian (end week 1)		
Visit – UHSM. Dietitian face to face review 1 (end week 2)	2	
Telephone review with dietitian (end week 3)	3	
Visit – UHSM: Dietician face to face review (end week 4). Asked to complete IPAQ for 7 previous days.	4	Dietary intervention period
Telephone review with dietitian (end week 5)	5	
Visit – UHSM. Dietitian face to face review 1 (end week 6)	6	
Visit - WMIC and The Christies (week 7): MR imaging, OGTT, biochemical tests and review with UHSM dietitian	7	
Visit - WMIC and The Christies: (week 8) MR imaging, OGTT, biochemical tests		
Final Visit – UHSM (week 8): Measures of REE, body fat, fat free mass, anthropometrics. Review of 7 day food records and IPAQ activity questionnaire. Given moving on diet and exercise advice (IER or DER) and offered 2 further monthly review appointments with dietitian	8	

Figure 5.1: Study outline.

5.2 BRRIDE-2 RECRUITMENT

Between January, 2015, and October 2015, a total of 28 participants were enrolled in this trial and randomly assigned to dietary intervention (Figure 5.2). Baseline characteristics of the two intervention groups at randomisation were similar (Table 5.1). There were five withdrawals from the IER group, and three in the DER group. The main reason for dropout was participant choice not to complete the dietary intervention. Characteristics of the per-protocol groups were comparable (Table 5.2).
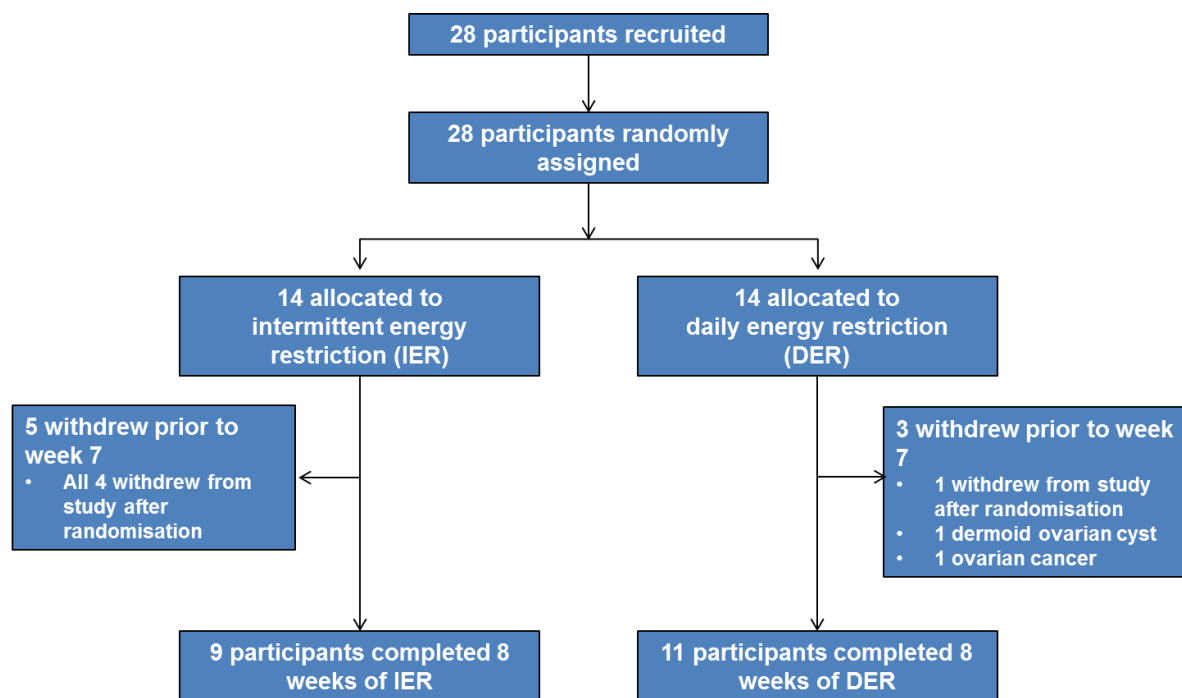
Figure 5.2: CONSORT diagram for BRRIDE-2 study.

Table 5.1: Baseline characteristics of intention-to-treat randomised participants

| | | Dietary Intervention | | p* |
		IER	DER	
n		14	14	
		Median (IQR)		
Age (years)		41 (37 – 42)	43 (39 – 47)	0.11
Height (m)		1.67 (1.63 – 1.69)	1.66 (1.59 – 1.71)	0.82
Weight (kg)		90.3 (85.7 – 104.1)	91.9 (84.7 – 103.1)	0.74
BMI (kg/m^2)		34.0 (31.1 – 38.3)	32.9 (31.3 – 36.6)	0.73
Waist to hip ratio		0.93 (0.91 – 0.98)	0.93 (0.89 – 0.99)	0.49
WC (cm)		113 (107 – 121)	111 (101 – 121)	0.63
Blood Pressure (mmHg)	Systolic	122 (116 – 133)	126 (119 – 137)	0.49
	Diastolic	79 (75 – 86)	83 (78 – 88)	0.63
Heart rate (bpm)		67 (59 – 71)	66 (62 – 70)	0.98
Resting Energy Expenditure (kcal/day)		1598 (1394 – 1902)	1454 (1333 – 1657)	0.18
Insulin (pmol/l)		78 (65 – 93)	75 (50 – 116)	0.78
C-peptide (ng/ml)		2.8 (2.7 – 3.5)	2.8 (2.2 – 3.3)	0.41
Fasting Glucose (mmol/l)		4.9 (4.7 – 5.2)	4.8 (4.6 – 4.9)	0.21
HOMA2-IR		1.6 (1.3 – 1.7)	1.4 (1.0 – 2.2)	0.62
HOMA2 %B		130 (115 – 142)	130 (108 – 165)	0.84
MRS measured anthropometrics	Hepatic fat (%)	3.16 (1.81 – 4.16)	3.45 (1.58 – 5.60)	0.66
	Pancreatic fat (%)	3.91 (1.91 – 6.17)	2.92 (0.88 – 5.65)	0.52
	Intramuscular fat (%)	6.26 (4.69 – 8.24)	6.31 (5.67 – 8.56)	0.56
Fat free mass (kg)		53.0 (51.2 – 59.8)	53.0 (49.7 – 56.4)	0.61
Trunk fat (%)		37.5 (35.0 – 40.9)	38.9 (36.0 – 41.8)	0.70
Muscle mass (kg)		49.5 (48.3 – 56.8)	50.4 (47.2 – 53.5)	0.96

HOMA2-IR (insulin resistance) calculated using the updated version of the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/index.php) using fasting glucose and insulin from OGTTs.

HOMA2-%B (beta function) calculated using the updated version of the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/index.php) using fasting glucose and C-peptide from OGTTs.

*Mann-Whitney U test

Table 5.2: Baseline characteristics of participants completing dietary intervention (per-protocol participants)

		Dietary Intervention		p*
		IER	DER	
n		9	11	
		Median (IQR)		
Age (years)		41 (37 – 42)	39 (39 -47)	0.18
Height (m)		1.66 (1.63 – 1.67)	1.65 (1.58 – 1.71)	0.94
Weight (kg)		89.6 (86.1 – 97.4)	92.5 (85.9 – 107.1)	0.52
BMI (kg/m^2)		33 (32 – 35)	34 (32 – 40)	0.54
Waist to hip ratio		0.97 (0.91 – 1.01)	0.94 (0.89 – 1.00)	0.30
WC (cm)		113 (109 – 118)	104 (101 – 128)	0.71
Blood Pressure (mmHg)	Systolic	120 (116 – 131)	130 (115 – 137)	0.62
	Diastolic	78 (75 – 86)	82 (78 – 88)	0.45
Heart rate (bpm)		62 (59 – 69)	66 (61 – 70)	0.36
Resting Energy Expenditure (kcal/day)		1548 (1390 – 1666)	1521 (1364 – 1702)	0.97
Insulin (pmol/l)		89 (80 – 96)	99 (60 – 117)	0.91
C-peptide (ng/ml)		3.1 (2.7 – 4.1)	3.1 (2.5 – 4.4)	0.62
Fasting Glucose (mmol/l)		5.2 (4.8 – 5.2)	4.7 (4.6 – 4.9)	0.09
HOMA2-IR		1.7 (1.6 – 1.7)	1.7 (1.1 – 2.3)	1.00
HOMA2 %B		136 (115 – 147)	140 (121 – 180)	0.45
MRS measured anthropometrics	Hepatic fat (%)	1.83 (1.28 – 3.98)	4.9 (1.73 – 9.71)	0.24
	Pancreatic fat (%)	3.74 (2.64 – 4.24)	3.11 (1.91 – 9.11)	0.88
	Intramuscular fat (%)	5.67(4.32 – 8.87)	6.82 (5.67 – 8.57)	0.32
Fat free mass (kg)		52.1 (51.4 – 59.0)	53.3 (49.5 – 58.2)	0.97
Trunk fat		35.8 (34.9 – 37.4)	39.7 (36.3 – 42.6)	0.18
Muscle mass (kg)		49.4 (48.3 – 53.3)	50.6 (47.0 – 55.3)	0.47

HOMA2-IR (insulin resistance) calculated using the updated version of the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/index.php) using fasting glucose and insulin from OGTTs.
HOMA2-%B (beta function) calculated using the updated version of the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/index.php) using fasting glucose and C-peptide from OGTTs.
*Mann-Whitney U test

5.3   DIETARY INTERVENTION

Dietary adherence was assessed by analysis of caloric intake from the second, fourth and eighth weeks of dietary intervention. Daily caloric requirements are calculated at baseline using measurement of resting energy expenditure (REE) multiplied by 1.3. The target overall weekly reduction in caloric intake for both diets was 25%. Participants on both diets tended to have greater calorie restriction than intended and maintained restriction throughout the trial (Figure 5.3), median weekly restriction for participants completing the trial was -32% (IQR -28 to -41). Although there was a trend to greater calorie restriction in the DER group, this was not significant (Table 5.3).
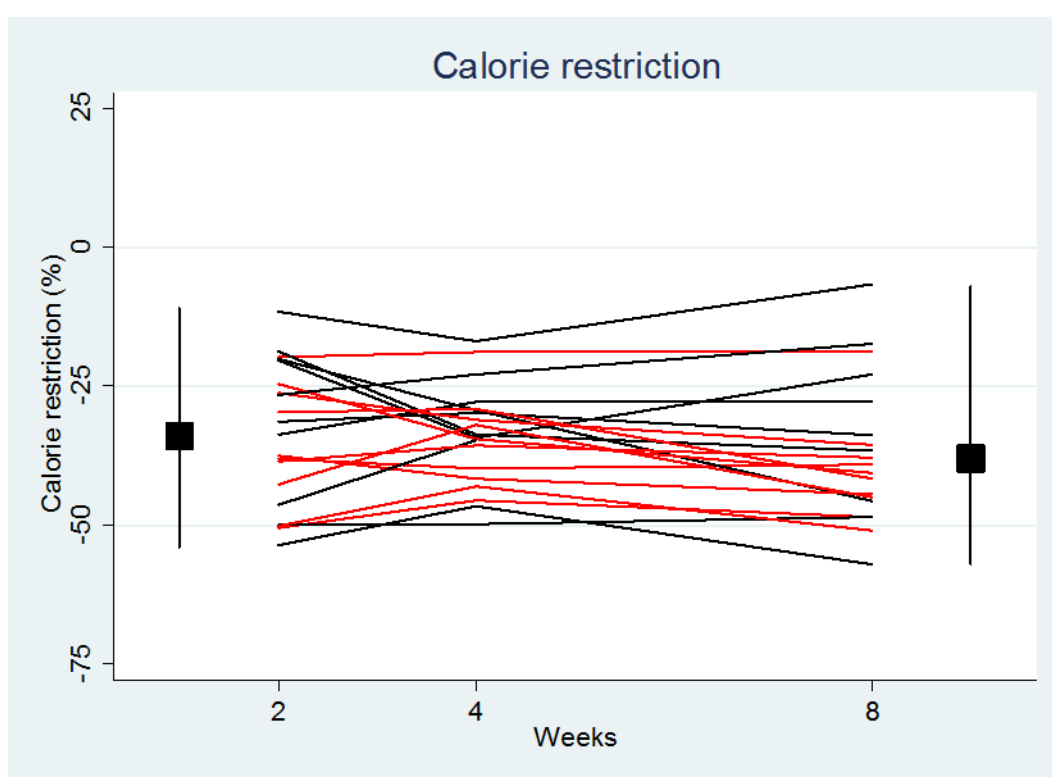
Figure 5.3: Calorie restriction. Each line represents an individual on either the Intermittent Energy Restriction (black lines) or Daily Energy Restriction (red lines) dietary intervention. The dotted line represents target calorie restriction (25% reduction). Box and whisker plots represent median and range of calorie restriction in week 2 (left) and week 8 (right) and demonstrate that participants maintained calorie restriction throughout the trial.

Table 5.3: Energy restriction by dietary intervention group.

	Energy restriction (percent) Median (IQR)			Mean weeks 2-8 restriction[1]	
	Week 2	Week 4	Week 8	Median (IQR)	p*
IER	-26 (-20 to -38)	-33 (-29 to -35)	-35 (-20 – -40)	-31 (-24 to -32)	0.06
DER	-38 (-30 to -50)	-36 (-29 to -43)	-43 (-32 – -49)	-39 (-30 to -48)	

*Mann-Whitney U test to compare median of mean (week 2-8) restriction between groups.
[1]Only participants completing trial included (n=20).

5.4   CLINICAL ENDPOINTS

The primary and secondary endpoints are listed in Table 5.4 and 5.5. Significant reductions in almost all measurements of excess body weight were observed in both groups.

5.4.1   CHANGES IN WEIGHT, BODY COMPOSITION AND METABOLIC MARKERS

Weight loss was comparable between the groups, weight reduced from median (IQR) 89.2 (86.1 – 97.4) to 81.8 (79.3 – 90.4) kg in the IER group compared with a reduction from 92.5 (85.9 – 107.1) to 87.8 (80.6 – 100.9) kg in the DER group (figure 5.4). Both groups experienced comparable reductions in BMI, waist circumference and fat-free mass but not WHR.
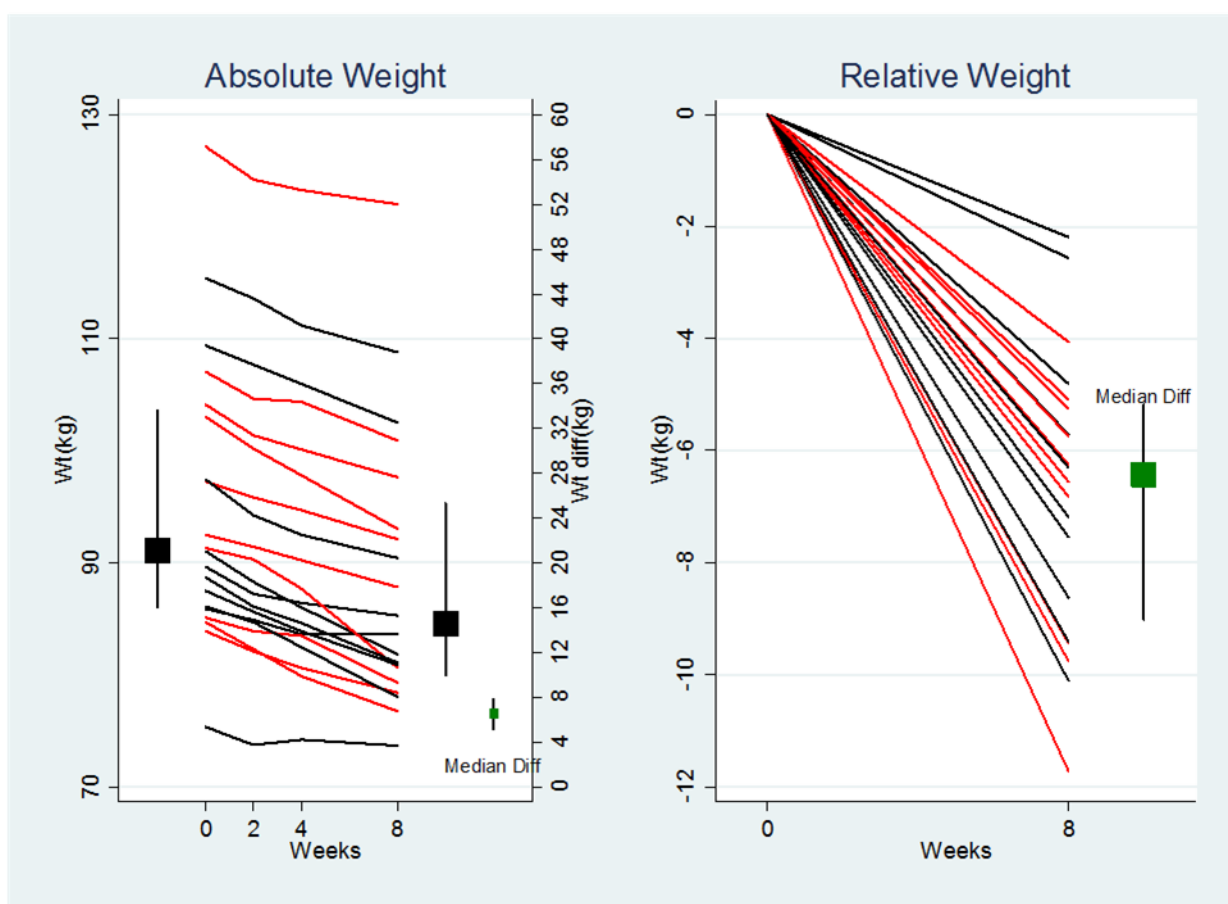
Figure 5.4: Absolute weight change (left) and relative weight loss (right). Each line represents an individual on either the Intermittent Energy Restriction (black lines) or Daily Energy Restriction (red lines) dietary intervention.

Table 5.4: Change in anthropometrics and MR-derived ectopic fat deposits before and after eight-week dietary intervention.

Parameter		Baseline	Week 8	p*
		Median (IQR)		
Weight (kg)				
IER		89.6 (86.1 – 97.4)	81.8 (79.3 – 90.4)	0.008
DER		92.5 (85.9 – 107.1)	87.8 (80.6 – 100.9)	0.003
WC (cm)				
IER		113 (109 – 118)	107 (106 -111)	0.008
DER		104 (101 – 128)	113 (98 – 123)	0.003
Blood Pressure (mmHg)	Systolic			
IER		120 (116 – 131)	117 (109 – 120)	0.015
DER		130 (115 – 137)	116 (111 – 126)	0.026
	Diastolic			
IER		78 (75 – 86)	78 (73 – 81)	0.028
DER		82 (78 – 88)	74 (73 -81)	0.007
Heart rate (bpm)				
IER		62 (59 – 69)	62 (60 – 64)	0.68
DER		66 (61 – 70)	62 (57 – 74)	0.53
MRS measured anthropometrics	Hepatic fat (%)			
IER		1.83 (1.28 – 3.98)	0.67 (0.13 – 1.66)	0.0007
DER		4.9 (1.73 – 9.71)	1.75 (0.72 – 3.95)	0.006
	Pancreatic fat (%)			
IER		3.74 (2.64 – 4.24)	1.64 (1.05 – 1.99)	0.009
DER		3.11 (1.91 – 9.11)	2.66 (0.54 – 5.62)	0.01
	Intramuscular fat (%)			
IER		5.67(4.32 – 8.87)	5.04 (3.98 – 7.36)	0.09
DER		6.82 (5.67 – 8.57)	6.53 (5.93 – 8.42)	0.78
Fat-free mass (kg)				
IER		52.1 (51.4 – 59.0)	50.6 (49.6 – 55.7)	0.008
DER		53.3 (49.5 – 58.2)	51.9 (47.7 – 55.6)	0.004
Trunk fat				
IER		35.8 (34.9 – 37.4)	35.3 (31.5 – 36.4)	0.008
DER		39.7 (36.3 – 42.6)	37.9 (34.2 – 40.2)	0.11
Muscle mass				
IER		49.4 (48.3 – 53.3)	48.1 (47.1 – 52.9)	0.11
DER		50.6 (47.0 – 55.3)	49.3 (45.3 – 56.4)	0.037

*Wilcoxan sign-rank test

Table 5.5: Change in metabolic markers before and after eight-week dietary intervention.

Parameter		Baseline	Week 8	Between interventions p*	Within interventions p†
		Median (IQR)			
Insulin (pmol/l)	Both interventions	91 (69 – 115)	68 (62 – 93)		0.12
	IER	89 (80 – 96)	76 (67 – 98)	0.25	0.44
	DER	99 (60 – 117)	63 (59 – 73)		0.15
C-peptide (ng/ml)	Both interventions	3.1 (2.7 – 4.3)	2.8 (2.4 – 3.1)		0.00
	IER	3.1 (2.7 – 4.1)	2.8 (2.6 – 3.0)	0.71	0.086
	DER	3.1 (2.5 – 4.4)	2.7 (2.0 – 3.1)		0.013
Fasting Glucose (mmol/l)	Both interventions	4.8 (1.6 – 5.2)	4.8 (4.5 – 5.1)		0.20
	IER	5.2 (4.8 – 5.2)	5.0 (4.5 – 5.1)	0.87	0.038
	DER	4.7 (4.6 – 4.9)	4.8 (4.6 – 4.9)		0.76
120 minute Glucose (mmol/l)	Both interventions	5.9 (4.8 – 6.9)	5.2 (4.8 – 6.0)		0.08
	IER	6.0 (4.8 – 6.5)	5.2 (5.1 – 5.8)	0.71	0.21
	DER	5.8 (4.8 – 7.0)	5.2 (4.8 – 6.2)		0.28
HOMA2-IR	Both interventions	1.6 (1.1 – 1.8)	1.3 (1.1 – 1.6)		0.03
	IER	1.7 (1.6 – 1.7)	1.4 (1.2 – 1.7)	0.20	0.44
	DER	1.7 (1.1 – 2.3)	1.3 (1.0 – 1.4)		0.02
HOMA2-%B	Both interventions	129 (109 – 151)	119 (106 – 146)		0.08
	IER	136 (115 – 147)	143 (107 – 150)	0.22	0.67
	DER	140 (121 – 180)	117 (95 – 136)		0.03

Complete serum biomarker data for both time points in 19 participants: 10 in the IER group; 9 in the DER group.

HOMA2-IR (insulin resistance) calculated using the updated version of the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/index.php) using fasting glucose and insulin from OGTTs.

HOMA2-%B (beta function) calculated using the updated version of the HOMA calculator (www.dtu.ox.ac.uk/homacalculator/index.php) using fasting glucose and C-peptide from OGTTs.

*Mann-Whitney test at week 8 (unadjusted)

†Wilcoxan sign-rank test

5.4.2 CHANGES IN ECTOPIC FAT

A key question in the study design was to assess changes in ectopic fat deposition after eight-weeks of dietary intervention and to identify if the 48 fast, for participants on the IER diet, was associated with a nadir in hepatic or pancreatic fat. To test this, MR measurements were obtained from participants in the IER group following the 48 hour fast in week seven and again at eight weeks after at least 48 hours of isocaloric dietary intake. As a control, participants in the DER group also had repeated measurement of ectopic fat in week seven and eight.

Overall, a median (IQR) 67 (39 – 87) % reduction in hepatic fat was observed with a 33 (11 -59) % reduction in pancreatic fat, these changes in hepatic and pancreatic fat were similar between the two groups. Between weeks seven and eight a trend to reduction in hepatic and pancreatic fat were observed for both groups but his was not significant (table 5.6). Four of ten participants in the IER group had greater hepatic fat in week eight than following fasting in week seven compared with two of ten participants in the DER group.
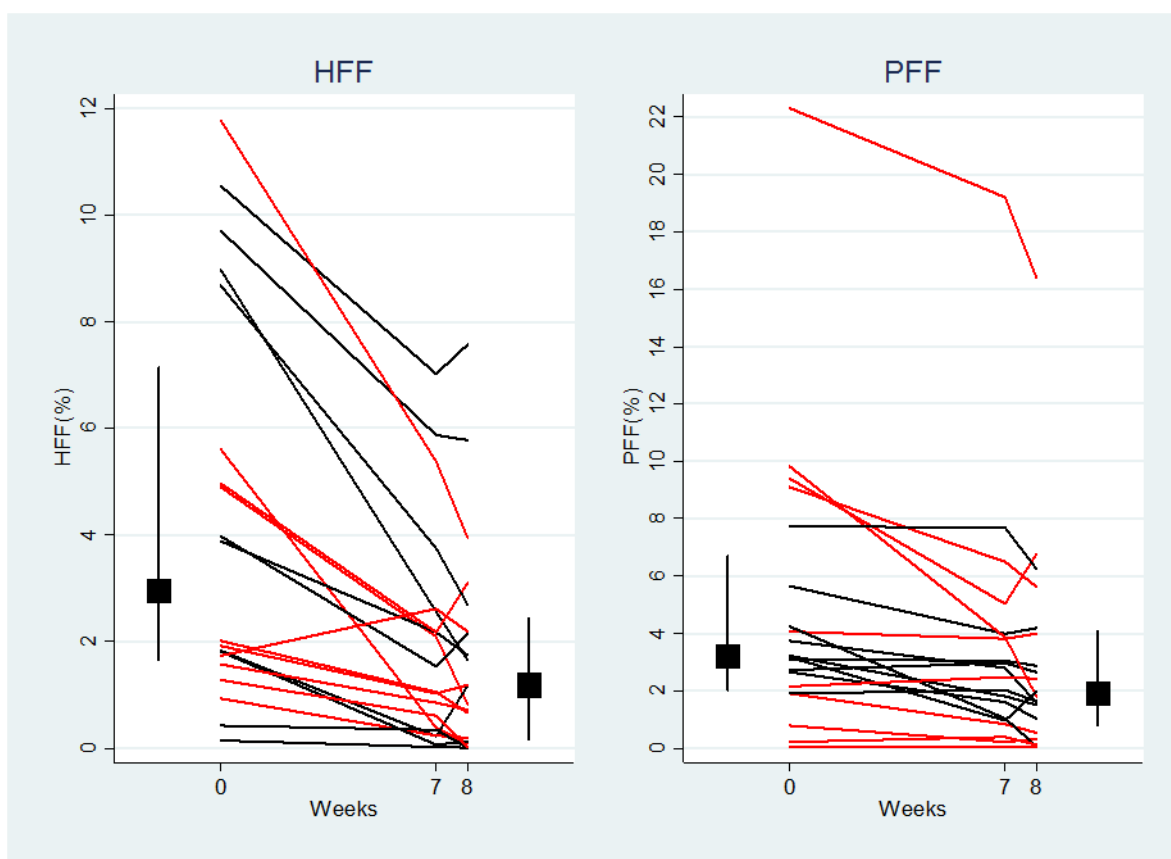
Figure 5.5: Changes in hepatic and pancreatic fat. Each line represents an individual on either the Intermittent Energy Restriction (black lines) or Daily Energy Restriction (red lines) dietary intervention. Box and whiskers represent median and range of fat at baseline (left) and week eight (right) or each graph.

Table 5.6: Hepatic and pancreatic fat at baseline, week seven and week eight.

	Baseline	Week 7	Week 8	Week 7 to week 8	Baseline to week 8
	Median (IQR)			p*	
Hepatic fat (%)					
IER	1.83 (1.28 – 3.98)	0.61 (0.24 – 1.54)	0.67 (0.13 – 1.66)	0.44	0.008
DER	4.9 (1.73 – 9.71)	2.17 (0.85 – 5.38)	1.75 (0.72 – 3.95)	0.29	0.006
Pancreatic fat (%)					
IER	3.74 (2.64 – 4.24)	2.02 (1.05 – 3.82)	1.64 (1.05 – 1.99)	0.10	0.009
DER	3.11 (1.91 – 9.11)	2.98 (0.85 – 5.03)	2.66 (0.54 – 5.62)	0.13	0.01

*Wilcoxan signed-rank test.

Table 5.7: Actual and percentage change in hepatic fat from baseline to week eight and between week seven and week eight.

		Between baseline and 8 weeks		
		Absolute difference	Relative difference (%)	
		Median (IQR)	Median (IQR)	P value*
Weight (kg)	Both interventions	6.6 (5.1 – 7.8)	6.4 (5.2 – 9.0)	0.0001
	IER	6.6 (5.8 – 7.0)	6.8 (6.3 – 7.5)	0.008
	DER	6.1 (4.7 – 8.0)	5.7 (4.1 – 9.4)	0.003
Hepatic fat (%)	Both interventions	1.76 (0.73 – 4.01)	67 (39 – 87)	0.0001
	IER	1.35 (0.73 – 1.83)	78 (67 – 93)	0.0007
	DER	2.14 (0.73 – 4.08)	54 (38 – 83)	0.006
Pancreatic fat (%)	Both interventions	1.41 (0.16 – 2.40)	33 (11 – 59)	0.0003
	IER	1.47 (0.27 – 2.15)	37 (14 – 60)	0.009
	DER	1.37 (0.08 – 2.64)	28 (7 – 53)	0.01
HOMA2-IR	Both interventions	0.3 (0.1 – 0.7)	18 (0 – 38)	0.12
	IER	0.3 (0.7 – 1)	16 (-35 - 87)	0.44
	DER	0.3 (0 – 0.7)	18 (-39 - 9)	0.02
HOMA2-%B	Both interventions	16.8 (18.5 – 96.3)	14 (6 – 34)	0.053
	IER	5.8 (-96.3 32.4)	0.0 (-42 – 11)	0.67
	DER	19.6 (-60.3 to -14.1)	15 (-22 to – 7)	0.03

HOMA2-IR and HOMA2-%B derived as in earlier tables.

Complete imaging data in 20 patients; complete serum biomarker data for both time points in 19 participants: 10 in the IER group; 9 in the DER group.

*Wilcoxan sign-rank test

5.4.3 TREATMENT EFFECT

As it is reasonable to expect that the starting weight, BMI, intra-hepatic fat, intra-pancreatic fat, and HOMA-IR influences final measurement, it would not have been appropriate to perform a direct comparison of week eight final measurements by group using either a t-test of Mann Whitney U. Instead I used a mixed-effects model repeated measures (MMRM) approach to test for the impact of treatment on the changes in measures of weight, BMI, HFF, PFF and HOMA-IR with time, while retaining within person correlation. In this setting, this approach was preferred (over ANOVA) as the variance of the data was unequal (see dotplots, figure 5.6). I used a 2 level model, to include time and person as levels, and with treatment as a covariate. We set up an initiator and then fit the model using adaptive quadrature, using the STATA `gllamm` command. Results of this analysis demonstrate that time was a strong predictor of reducing weight, BMI, hepatic and pancreatic fat. For each dependent variable the model that included individuals as an additional level (model 2, table 5.8) explained a greater amount of the variance.

Table 5.8: Results of a mixed-effects model repeated measures approach to test for impact of treatment on weight, hepatic fat, pancreatic fat, HOMA2-IR and HOMA2-%B.

Dependent variable	Levels & covariates*	β Co-efficient (95% Confidence interval)	p
Weight (kg)	Time (as a level)	-0.78 (-0.70 to -0.90)	< 0.001
	Treatment (as covariate)	-4.80 (-15.15 to 5.54)	0.36
Hepatic fat (%)	Time (as a level)	-0.32 (-0.23 to -0.41)	< 0.001
	Treatment (as covariate)	1.01 (-1.05 to 3.07)	0.34
Pancreatic fat (%)	Time (as a level)	-0.21 (-0.13 to-0.30)	< 0.001
	Treatment (as covariate)	-1.67 (-5.23 to 1.88)	0.36
HOMA2-IR	Time (as a level)	-0.03 (-0.09 to 0.02)	0.25
	Treatment (as covariate)	0.23 (-0.28 to 0.74)	0.38
HOMA2-%B	Time (as a level)	-6.1 (-9.5 to -2.6)	< 0.001†
	Treatment (as covariate)	5.3 (-62.5 to 73)	0.878

Treatment: 1 = IER; 0 = DER.
Time as a linear equation.
*All models were two-level: level 1 = individual person; level 2 = time. In all models, a random intercept was used.
†This significance was driven by the low median values at 7 weeks (which might have occurred by chance).
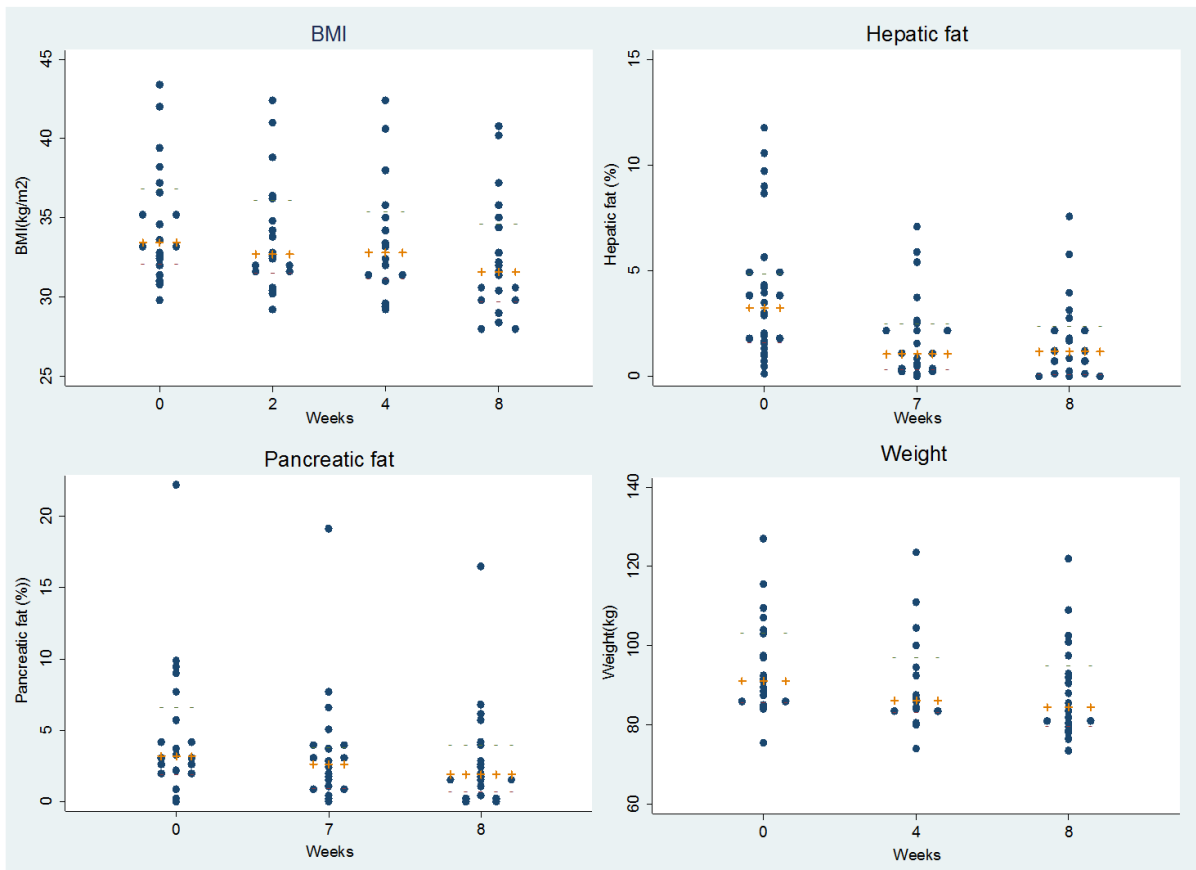
Figure 5.6: Dotplots to demonstrate unequal variance for repeated measurements of BMI, hepatic fat, pancreatic fat and weight. Yellow horizontal dots represent median, green horizontal dots inter-quartile range.

5.5 RELATIONSHIPS BETWEEN CHANGES IN MEASUREMENTS OF EXCESS ADIPOSITY

A final question I wanted to ask was whether changes in anthropometric measurements i.e. weight, WC and BMI were closely linked to changes in hepatic and pancreatic fat. As starting hepatic fat or pancreatic fat was associated with the actual loss in intra-hepatic or intra-pancreatic fat I tested relationships between percentage change in anthropometric indices. There were no significant relationships between percentage change in intra-hepatic or intra-pancreatic fat and percentage change in weight, waist circumference or BMI (table 5.9). Figures 5.7 and 5.8 demonstrate the variation in ectopic fat loss for participants on this trial.

For both percentage change in intra-hepatic fat and percentage change in intra-pancreatic fat, linear regression analysis confirmed that percentage change in BMI, weight and waist circumference did not predict percentage change in intra-hepatic or intra-pancreatic fat (table 5.10).

Table 5.9: Relationship between percentage change from baseline to week 8 in anthropometric and MR measured indices. Each row shows values for rho (top) and p (bottom).

	Δ Hepatic fat	Δ Pancreatic fat
Δ Pancreatic fat	0.105 0.659	
Δ BMI	0.378 0.100	0.298 0.202
Δ Weight	0.391 0.088	0.308 0.186
Δ Waist circumference	0.236 0.316	0.164 0.490

Table 5.10: Results of linear regression analysis to determine predictors of percentage change in ectopic fat deposits.

Dependent variable	Independent variable	β Co-efficient (95% Confidence interval)	p*
Δ Hepatic fat	**Δ** BMI	12.6 (-155.4 to 180.6)	0.876
	Δ Weight	-7.7 (-171.7 to 156.3)	0.922
	Δ WC	-1.2 (-8.6 to 6.3)	0.744
Δ Pancreatic fat	**Δ** BMI	8.9 (-147.3 to 165.0)	0.906
	Δ Weight	-2.6 (-155.0 to 149.8)	0.972
	Δ WC	-2.3 (-9.2 to 4.6)	0.481
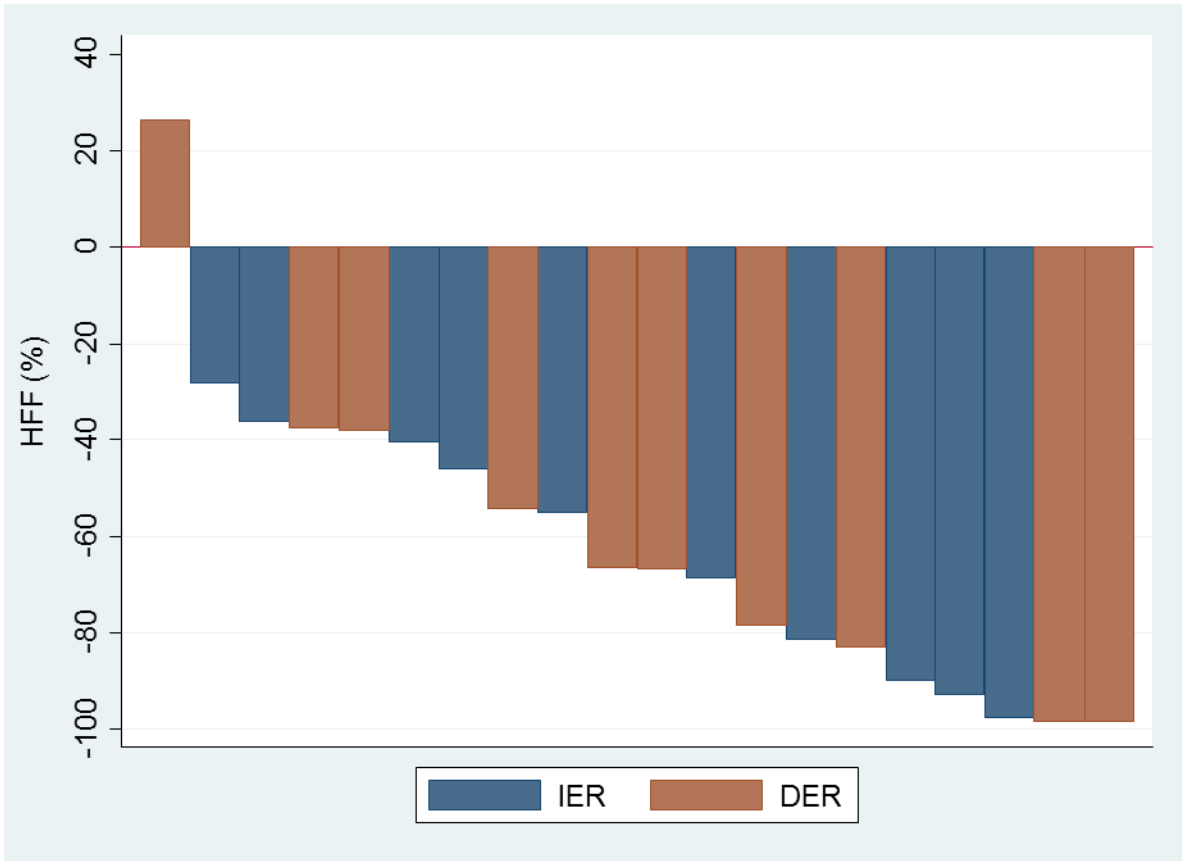
Figure 5.7: Waterfall plots of HFF percent change (from baseline to 8 weeks) according to intervention (assuming IER = 1; and DER = 2). One can see: (i) almost all individuals have a reduction in HFF after weight loss intervention; (ii) but that there is a wide variation in HFF percent change; and (iii) that the changes are seen for both IER and DER.
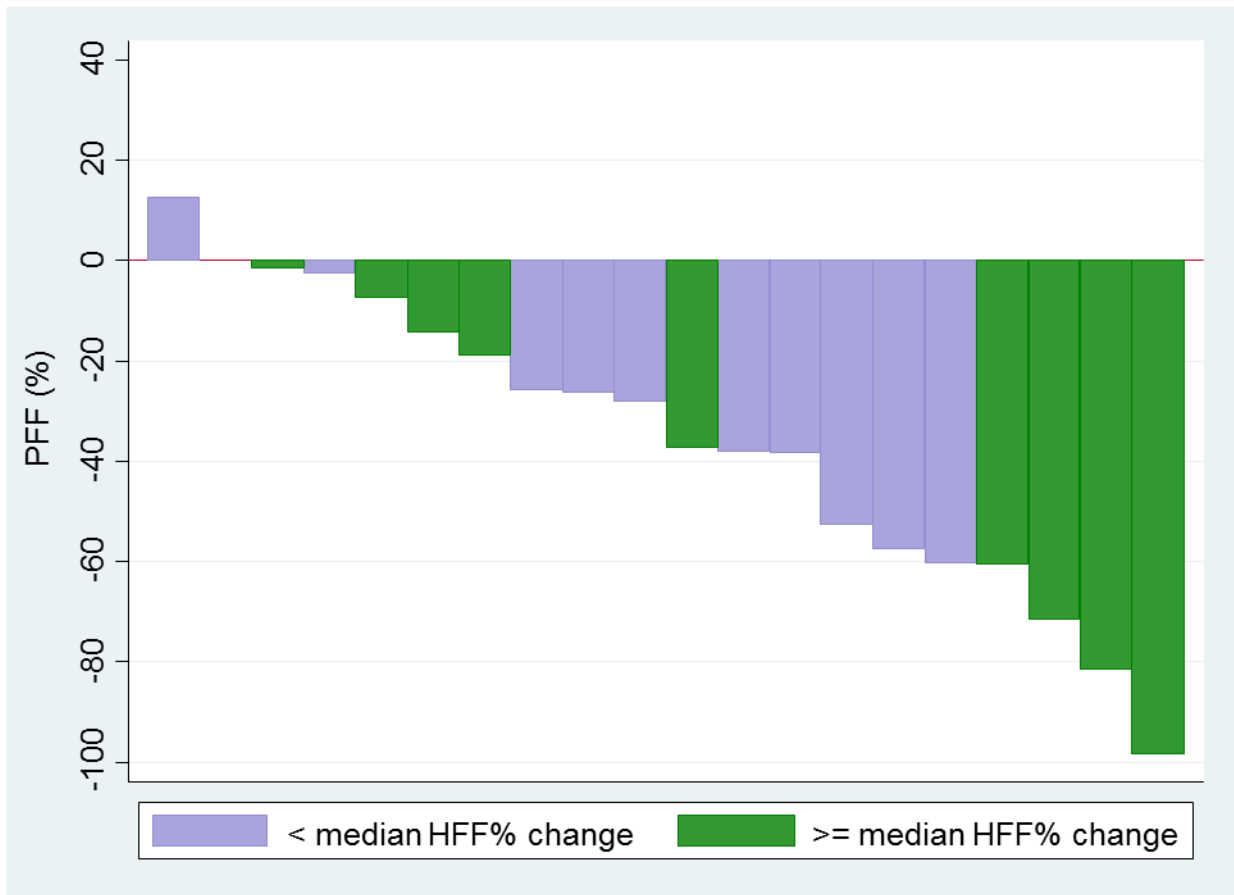
Figure 5.8: Waterfall plots of PFF percent change (from baseline to 8 weeks) – according to HFF percent changes (dichotomised above and below the median change, minus 66%). One can see: (i) almost all individuals have a reduction in PFF after weight loss intervention; (ii) but that there is a wider variation in PFF percent change; (iii) compared with HFF% changes (median change = -66%), median reduction of PFF % change is less (-33%); and (iv) that among individuals with a high HFF% reduction, there are some (green colour to right) with a corresponding large reduction in PFF%, but equally a group of individuals (green colour on left) where there is a good reduction in HFF% but relative resistance to PFF reduction.

5.6 DISCUSSION

5.6.1 MAIN FINDINGS

- Eight-weeks of dietary intervention in the form of 25% calorie restriction with either IER or DER are associated with a greater than 60% reduction in intra-hepatic fat. There was no difference in the effect on hepatic fat between these two methods of dieting.

- Intra-pancreatic fat can be reduced with dietary restriction. Calorie restriction with either IER or DER is associated a greater than 30% reduction in intra-pancreatic fat after eight-weeks; intra-pancreatic fat deposition is modifiable.

- After seven weeks of dieting, the 48 fast in the IER group was associated with a hepatic fat nadir in four of ten participants. This was not significantly different from the DER group.

- A greater proportion of intra-hepatic fat is lost during dietary intervention than intra-pancreatic fat but the degree of hepatic and pancreatic fat loss does not correlate with loss of weight or BMI. This may indicate that intra-hepatic fat is more easily mobilized by the body for use as an energy store than intra-pancreatic fat.

5.6.2 COMPARISON WITH THE LITERATURE

This is the first study to compare the effect of IER with DER on ectopic fat stores. Previous research into the effects of IER have focused on anthropometric (weight, BMI) and biochemical improvements. The predecessor to this study was a randomized controlled trial of 107 overweight premenopausal women.[188] Harvie et al demonstrated an equivalent effect for IER (~2710 kj/day) with DER (~6276 kj/day) on weight loss, fasting glucose, and biochemical markers of inflammation (IGF-1, high-sensitivity CRP). A greater reduction in fasting insulin and insulin resistance (HOMA) was seen with IER than with DER.

Previous to this major study, only two other small randomised studies comparing IER with DER are published. Hill et al demonstrated greater reductions in cholesterol (14 vs 6%) in the IER group when comparing alternating weeks of 2508, 3762, 5016 or 7254 kJ/day with with DER of 5016 kJ/day in 16 moderately obese women.[234] Ash et al compared an IER (4180 kJ liquid very low carbohydrate diet 4 days per week, 3 days ad libitum) with DER (6000–7000 kJ/day) in nine men with type 2 diabetes and showed no difference in terms of weight or fasting insulin.[235]

A larger volume of literature has investigated the effect of reduced daily energy intake dietary intervention on intra-hepatic fat. Browning et al.[210] compared carbohydrate restriction (<20 g/day) with low calorie dieting (5160 kJ/day women and 6465 kJ/day men) and showed a greater reduction in intra-hepatic fat with carbohydrate restriction group (55% vs 28%) in patients with Non Alcoholic Fatty Liver Disease (NAFLD) and a mean BMI of 35 kg/m^2. Lim et al.[179] investigated the effect of eight-weeks of 600 kcal/day dieting on intra-hepatic fat in patients with Type II diabetes and found a 79% reduction with 30% occurring in the first week of dietary intervention. Kirk et al.[205] compared the effect of low-fat high-carbohydrate dieting with low-carbohydrate dieting on hepatic fat in obese subjects and found greater reductions after 48 hours of dieting in the low-carbohydrate group (29.6% vs 8.9%) but equivalent reductions (38% vs 44.5%) once 7% weight loss was achieved. Ryan et al.[236] compared six-weeks of Mediterranean diet with a low-fat high-carbohydrate diet in a cross-over design. Despite similar weight loss, greater hepatic fat loss was observed for participants on the Mediterranean diet (39% vs 7%). Hollingsworth et al.[237] investigated the effect of a low carbohydrate diet on intra-hepatic fat in 10 participants and found 3kg weight loss was associated with a 11 to 43% loss of intra-hepatic fat. Similarly, Rossi et al. investigated the effect of hypocaloric (500kcal below resting energy expenditure daily restriction) dietary intervention and demonstrated an 84.1% reduction in intra-hepatic fat.[178] Further studies have investigated the effect of dietary composition on intra-hepatic fat using isocaloric diets. Three studies have demonstrated decreased fat intake despite equivalent overall calories is associated with loss of intra-hepatic fat.[238-240] Subdivision of dietary fat intake indicates that poly-unsaturated fatty acids may be associated with reduced intra-hepatic fat when compared with saturated fatty acids.[241] and mono-unsaturated fatty acids when reduce intra-hepatic fat when compared with isocaloric high-carbohydrate, high-fibre, low-glycaemic index dieting.[211]

A much smaller volume of literature has investigated the effect of weight loss interventions on intra-pancreatic fat. Two studies of dietary intervention and one of bariatric surgery have reported that weight loss leads to a reduction in intra-pancreatic fat. Rossi et al measured the effect of a 500kcal below resting energy expenditure diet on intra-pancreatic fat in 24 obese (mean BMI 35.4 kg/m^2) adults. Participants remained on the diet until greater than 7% of initial weight was lost. A mean body weight decrease of 8.9% was associated with a 42.3% reduction in intra-pancreatic fat.[178] Lim et al, studied eleven obese (mean BMI 33.6 kg/m^2) people with type II diabetes on an eight-week severe calorie restricted diet (600kcal/day) and found a 15% reduction in body weight associated with a reduction in intra-pancreatic fat of 23% and improvements in insulin suppression of hepatic glucose output.[179] Finally, a 44% reduction in intra-pancreatic fat and 25% reduction in body weight was observed in 20 patients following bariatric surgical intervention.[180] Two studies, described

above, investigated the effect of dietary intervention on both hepatic and pancreatic fat. Rossi et al.[178] and Lim et al.[179] found greater reductions in intra-hepatic fat (84% and 79%) than intra-pancreatic fat (42% and 23%).

5.6.3 STRENGTHS AND WEAKNESSES

The present study has a number of strengths. It is the first trial to compare head-to-head the effects of IER with DER on ectopic fat deposits. These are important findings as unlike more severe dietary intervention (for example the 70% daily reduction used by Lim et al[179]), this group has previously demonstrated that IER and DER have acceptable adherence at 6-months.[188] Secondly, we report significant changes in intra-hepatic fat (greater than 60%) and intra-pancreatic fat (greater than 30%) that are similar to previous studies investigating the effect of dietary intervention. Thirdly, the study design has a number of strengths; the two groups were well matched for baseline characteristics and diets were well matched for known confounders of intra-hepatic fat so dietary fat composition was equivalent and all participants were sedentary and did not increase exercise activity during the study; by using a multilevel model we were able to control for individual starting intra-hepatic fat and compare the effect of dietary intervention. Additionally, there were no significant differences in intra-hepatic or intra-pancreatic fat between groups at baseline and weight loss was similar. Finally, by measuring fasting ectopic fat at week seven, immediately after 2 days of severe energy restirction this study investigated the acute term effects of energy restriction on intra-hepatic and pancreatic fat after the initial rapid weight loss period.

The major weakness for this study is the potential that it is underpowered for its primary endpoint. When designing the study, a 15% relative difference between intra-hepatic fat was chosen based on differences observed in other studies. This was the first study to address the question of IER versus DER on intra-hepatic fat and so no prior data were available. The original BRRIDE study did demonstrate differences in HOMA and fasting insulin levels and I hypothesised that this would be due to a difference in intra-hepatic fat which we did not demonstrate here. Additionally, due to the intensive nature of the study, our drop-out rate was higher than expected and this reduced the power of our findings.

6 DISCUSSION

6.1 KEY FINDINGS FROM THREE STUDIES

At the outset of this research program, my hypothesis posited that intra-pancreatic fat quantity is a predisposition biomarker for the development of pancreatic cancer secondary to excess-adiposity. Cancer Research UK (CRUK) sets out clear guidelines for biomarker discovery and development. Potential biomarkers must go through a process of discovery and assay development followed by qualification. For intra-pancreatic fat, research into its clinical relevance was essentially non-existent or limited by the lack of a non-invasive test to quantify this ectopic fat store.

To test my hypothesis, therefore, there was the need to start at the beginning of the CRUK biomarker roadmap and assess methods of measuring intra-pancreatic fat. The magnetic resonance imaging techniques CS-MR and MRS are in development as methods to quantify intra-hepatic fat. However, just a handful of studies reported their use in the quantification of intra-pancreatic fat. Therefore, at the outset of this research I needed to address the first stage of biomarker and assay development, assessment of accuracy and precision (reproducibility).

Chapters three and four of this thesis detail results of two studies investigating the accuracy and precision of CS-MR and MRS. I established that both MRS and CS-MR are accurate and reproducible to measure intra-pancreatic fat, albeit with some caveats.

- In stage-one of the PanORAMA project (chapter 3), I extended our group's experience with histological quantification of intra-hepatic fat to the pancreas to allow histological quantification of intra-pancreatic fat and thus a reference standard with which to compare CS-MR and MRS results. Results of that study found that CS-MR and MRS had agreement with histological assessment of intra-pancreatic fat, but correlations were only moderate to good (rho 0.672 and 0.781 respectively). Both techniques over-estimated intra-pancreatic fat compared with histology. This is the only study to assess accuracy in a human population. For researchers in this field, the difficulty has been obtaining pancreatic tissue so previous validation studies compared MR results with pancreatic tissue analysis limited to animal models.[100]

- Stage-two of the PanORAMA project dealt directly with precision. Here, I demonstrated that CS-MR, and after refinement, MRS, have clinically acceptable precision. This study tested this principle in intra-pancreatic fat in healthy volunteers with a range of intra-pancreatic fat consistent with the literature on the healthy population. Additionally, within this study, I was able to move to biomarker qualification. I established that the

correlations between intra-pancreatic fat and other markers of excess adiposity is present but generally weak. This prompts the investigation of intra-pancreatic fat as a biomarker of pancreatic cancer risk more informative than anthropometric measures alone.

- The second major aim of this thesis was to establish if intra-pancreatic fat could be reduced with dietary intervention i.e. was modifiable. Here, I moved several steps forward in the CRUK roadmap to biomarker qualification. This is an important principle for future studies as potential risk may be modifiable and pancreatic cancer cases prevented. The BRRIDE-2 is the first study to investigate the effects of intermittent energy restriction verses daily energy restriction on intra-pancreatic and intra-hepatic fat. Overall, I found that significant reductions (mean: 6.5%) in both of these ectopic fat stores could be achieved with eight-weeks of dietary intervention. The two interventions, IER and DER, in the form prescribed to participants in the BRRIDE-2 trial, are well tolerated and have good adherence.[188] This is important as it establishes that efficacious dietary interventions are possible in a healthy population. Previous studies demonstrating this principle are limited to; populations undergoing bariatric surgery;[180] a study of severe (600kcal/day restriction) dietary restriction in participants with type II diabetes mellitus;[179] a single arm study of obese men.[178]

- In chapter 3 (on precision), I noted that the concordance between hepatic fat fraction and pancreatic fat fraction is incomplete. As an extension of this, the BRRIDE-2 trial found that the proportions of hepatic fat fraction reductions was imperfectly correlated with proportions of pancreatic fat fraction reductions, despite weight loss in all individuals after dietary intervention. In other words, some individuals had a substantial reduction in hepatic fat fraction and pancreatic fat fraction, but for other individuals, there was a substantial reduction in hepatic fat fraction but a *relative resistance* to pancreatic fat fraction reduction. This raises the hypothesis that there might be particular individuals at risk of cumulative adverse intra-pancreatic fat despite adequate attempts to lose weight.

Conventional thinking on biological mechanisms linking obesity and cancer risk, including pancreatic cancer risk, have been along three hormonal 'systemic' pathways: sex hormones; insulin and insulin-like growth factors; and adipokines and subclinical systemic inflammation. More recent hypotheses have focused on the important of within organ local ectopic fat as an abnormal micro-environment favouring cancer development and progression. This was elaborated in the Nature Reviews in Cancer review from Renehan and colleagues in 2015.[233] Importantly, this hypothesis explains the specificity of epidemiological associations between excess adiposity and cancer risk. The observations that within a given individual, in the presence of short-term weight

reduction, there are differential changes in local within organ fats – hepatic fat and pancreatic fat – supports the specificity hypothesis. In other words, within an obese individual, different organs may be at different disease risks from the differential accumulation of excess local fats.

6.2 GENERAL LIMITATIONS AND STRENGTHS

There were several thesis limitations (and things that if I had my time again, I might do differently). First, the set-up process for the BRRIDE-2 trial (chapter 5) took nearly 6 months reflecting the complexity of involvement of multiple disciplines and multiple institutes (University of Manchester; The Christie NHS Foundation Trust; and South Manchester University Hospitals NHS Trust) to effect the trial. Within a 2-year MD Thesis, it is challenging to undertake complex intervention trials, even with small samples sizes. Second, the PANORAMA studies (chapters 3 and 4) were unrestricted in terms of normal and overweight categories. For future healthy volunteer reproducibility studies, enhanced recruitment of overweight and obese individuals might deliver a wider range of fat-related values on imaging – ultimately being more statistically efficient. Third, in the BRRIDE-2 trial, in preparation, we anticipated a 20% drop-out rate – but this was an underestimate. We did not formally and systematically capture reasons for drop out (again a weakness, in retrospect), but subjectively, the main reason for trial withdrawal was pressure of time. There were two individuals who found the MR scanner claustrophobic and terminated the trial early. Finally, there was no difference in hepatic fat fraction between IER and DER after 8 weeks of dietary intervention. However, this was based on only ten participants per arm who completed the full trial. This lack of difference may be a type 2 statistical error i.e. the sample sizes were insufficient to show a statistical difference.

There were several general strengths in this thesis. First, the supporting team was multi-disciplinary including expertise in obesity-cancer science (Renehan); surgical and medical oncology of pancreatic cancer (O'Reilly, Valle); imaging science (Williams); cancer-related nutrition (Harvie); endocrinology (Higham) and biostatistics (Morris, Sperrin). Serum assays for insulin and C-peptide were undertaken in an internationally renowned laboratory in Aarhus University, Denmark (Frystyk). Second, there were opportunities to discuss the hypotheses and early results as national meetings and with visiting international experts in obesity-related pancreatic cancer development, for example, Professor Stephen Hurstings, from University of Austin, Texas, in June 2015 (animal models). This was important as the techniques were, in the main, new in the context of pancreatic cancer. Third, there was flexibility within the research programme to troubleshoot and repeat human experiments on order to modify and substantially improve the reproducibility of MRS for quantification on intra-pancreatic fat among healthy volunteers (as was done in chapter 4). Fourth, for the

assessment of insulin resistance and insulin secretion, I undertook dynamic testing using a modified oral glucose tolerance test (in BRRIDE-2). This approach is preferred to HOMA-IR only and preferred in the context of a dietary intervention trial.[242] Although, the present thesis did not allow me to complete in-depth analysis of the OGTT dynamics (due to time restrictions), I did report parameters at time zero and at 2 hours (the latter a key surrogate time for insulin resistance. Fifth, I measured several other obesity-related indicators such as hepatic fat fraction, VAT and SAT, and WC, allowing me to compare the relative 'correlations' of these with each other and with insulin resistance (albeit with small samples sizes). Finally, the central 'big' strength of the thesis was the BRRIDE-2 trial. Here, all participants who completed the trial has good adherence to dietary intervention and achieved weight loss allowing the study to demonstrate that pancreatic fat fraction (and other fat deposition indicators) are modified over short-term; that these changes were variable; and not necessarily well-correlated i.e. there was individual specificity of response.

6.3 FUTURE STUDIES ON INTRA-PANCREATIC FAT CONTENT

There is now a need to move forward from the biomarker development and assay phase to biomarker qualification stage 1 (CTAAC/BIDD BM qualification stage 1). It is necessary to skip biomarker discovery (BIDD BM discovery stage 2) as a retrospective analysis of the relationship between intra-pancreatic fat and pancreatic cancer development is not possible due to reverse causality i.e. intra-pancreatic fat is altered in the presence of pancreatic cancer. There is evidence that intra-pancreatic fat is linked with an intermediate marker of pancreatic cancer development, pancreatic intraepithelial neoplasms (PanINs).[143] However, the link between the presence of PanINs, their frequency and severity, and the subsequent development of pancreatic cancer is unknown. The French study by Rebours et al.[143, 243] is unique (and challenging to replicate elsewhere in the world) due to the large number surgically resected pancreas specimens, without pancreatic ductal adenocarcinoma, available to this group and could not be reproduced for pancreatic cancer due the reasons given above.

There are two avenues for further research that emerge from my findings. The first is to build on results from the BRRIDE-2 study. I now know that intra-pancreatic and intra-hepatic fat are differentially reduced after a weight reduction intervention. There are no data to distinguish responders from non-responders, so going forward, I would do a single arm larger weight reduction study with three parts:

Firstly, I would screen initially for moderate to high intra-hepatic fat. So this part of the study itself would have to develop a screening tool e.g. USS, HOMA-IR or serum fetuin A benchmarked against MRS.[244] I would then run a sufficiently large study to identify

individuals who are resistant to reduction in intra-pancreatic fat after weight reduction. Once this cohort is established (probably with 200 to 300 individuals), run global analyses (e.g. SNPs or whole genome analyses) to distinguish resistant and non-resistant groups.

The second set of studies would be to follow a more mechanistic route via imaging – exploring relationships between intra-pancreatic fat and functional endpoints. Since the target group is the healthy 'at-risk' population, human studies investigating the link between intra-pancreatic fat and pancreatic cancer development should be focussed here.

My hypothesis is of a pathological process analogous to the progression of non-alcoholic fatty liver disease (NAFLD) through Non-alcoholic steatohepatitis (NASH) to Hepatocellular carcinoma (HCC). The clinical condition of NASH is defined by the presence of inflammation on histological examination of liver biopsies but hepatic fibrosis is also considered important in progression to HCC. Therefore, to test the translation of this theory to pancreatic disease we must develop tests to quantify and classify pancreatic inflammation and fibrosis.

The first significant obstacle is that histological sampling of the pancreas cannot justifiably be performed in a healthy population. An early priority must therefore be to translate non-invasive tests of inflammation and fibrosis for assessment of the healthy pancreas. Here, there is the possibility to borrow from other comparable research fields such as the hepatic literature as well as research teams investigating the autoimmune destruction of the pancreas.

The clinically approved magnetic nanoparticle (MNP) ferumoxytol has recently been assessed for use to image pancreatic inflammation in patients with type I diabetes mellitus.[245] This MNP-MRI approach reflects nanoparticle uptake by macrophages in the inflamed pancreatic and is validated in mouse models of type I diabetes mellitus and in a pilot human study.[246] A recent study has demonstrated clear difference in whole-pancreas nanoparticle accumulation in patients and controls and interestingly, significant inter- and intra-pancreatic variation in signal intensity.[245] If translated to the healthy pancreas, the ability to generate non-invasive maps of pancreatic inflammation presents an interesting opportunity to assess the association of intra-organ fat with nanoparticle accumulation but also to assess the response of pancreatic inflammation to dietary or pharmacological intervention in longitudinal studies.

A second important intermediary endpoint would be the development of fibrosis.[143] Here, non-invasive methods of assessing intra-hepatic fibrosis in the setting of NASH are being translated to the assessment of the pancreas.[247] Magnetic Resonance Elastography (MRE) directly visualizes and quantitatively measures acoustic shear waves progressing through the liver tissue.[248] Early results indicate that reproducible measurements can be

obtained for the pancreas.[247] However, validation of this technique requires comparison with pancreatic histology, a step not yet taken.

6.4 CONCLUSIONS

This is the first set of studies, to my knowledge, that has specifically addressed questions around using MR imaging quantification to characterise intra-pancreatic fat in the context of cancer risk predisposition.

CS-MR and MRS are both fit for purpose for quantifying pancreatic fat fraction. The greatest advantage of these modalities is that they are non-invasive and non-ionising radiation. The greatest disadvantage of the modalities is cost, limited availability and currently, are time-consuming.

Nonetheless, there has been near no advances in pancreatic cancer risk prediction or prevention in the last three decades. At the end of this thesis, I have developed a framework to extend the described work to a larger number of people and link with other researchers to explore the use of combining MR imaging biomarkers of pancreatic fat with, for example, gene studies. This will lead to the development of a "fingerprint" of biomarkers with which to better predict an individual's level of risk for pancreatic cancer, and ultimately prevent many cancer fatalities in the future.

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