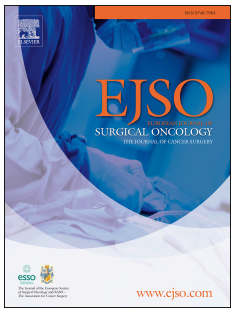
Accepted Manuscript



Digital Histology Quantification of Intra-hepatic Fat in Patients undergoing Liver Resection

Mr Ed Parkin, Derek A. O'Reilly, Andrew A. Plumb, Prakash Manoharan, Madhu Rao, Peter Coe, Jan Frystyk, Basil Ammori, Nicola de Liguori Carino, Rahul Deshpande, David J. Sherlock, Andrew G. Renehan

PII: S0748-7983(15)00434-5

DOI: 10.1016/j.ejso.2015.05.003

Reference: YEJSO 4063

To appear in: *European Journal of Surgical Oncology*

Received Date: 30 March 2015

Accepted Date: 7 May 2015

Please cite this article as: Parkin E, O'Reilly DA, Plumb AA, Manoharan P, Rao M, Coe P, Frystyk J, Ammori B, de Liguori Carino N, Deshpande R, Sherlock DJ, Renehan AG, Digital Histology Quantification of Intra-hepatic Fat in Patients undergoing Liver Resection, *European Journal of Surgical Oncology* (2015), doi: 10.1016/j.ejso.2015.05.003.

Digital Histology Quantification of Intra-hepatic Fat in Patients undergoing Liver Resection

Ed Parkin,[1,2] Derek A O'Reilly,[1,2] Andrew A Plumb,[3] Prakash Manoharan,[3] Madhu Rao,[4] Peter Coe,[1,2] Jan Frystyk,[5] Basil Ammori,[2] Nicola de Liguori Carino,[2] Rahul Deshpande,[2] David J Sherlock,[2] Andrew G Renehan[1,6]

1 Institute of Cancer Sciences, University of Manchester, Manchester, UK
2 Department of Hepatobiliary Surgery, North Manchester General Hospital, Manchester, UK
3 Department of Radiology, The Christie Hospital NHS Foundation Trust, Manchester, UK
4 Department of Histopathology, Royal Oldham Hospital, Oldham, UK
5 Medical Research Laboratory, Department of Clinical Medicine, Aarhus University, Denmark
6 Department of Colorectal Surgery, The Christie Hospital NHS Foundation Trust, Manchester, UK

Correspondence to:
Mr E Parkin
Institute of Cancer Sciences
University of Manchester
Manchester Academic Health Science Centre
The Christie NHS Foundation Trust
Wilmslow Road
Manchester M20 4BX United Kingdom
Tel: +44 161 446 3157
E-mail: ed.parkin@nhs.net

(Word count - excluding title page, abstract, references, figures and tables)

Abstract: 250 words (max: 250); main text: 2705 words; 3 tables; 2 figures; supplemental material (5 pages); 32 references; language, U.K. English

ABSTRACT

Background: High intra-hepatic fat (IHF) content is associated with insulin resistance, visceral adiposity, and increased morbidity and mortality following liver resection. However, in clinical practice, IHF is assessed indirectly by pre-operative imaging [for example, chemical-shift magnetic resonance (CS-MR)]. We used the opportunity in patients undergoing liver resection to quantify IHF by digital histology (D-IHF) and relate this to CT-derived anthropometrics, insulin-related serum biomarkers, and IHF estimated by CS-MR.

Methods: A reproducible method for quantification of D-IHF using 7 histology slides (inter- and intra-rater concordance: 0.97 and 0.98) was developed. In 35 patients undergoing resection for colorectal cancer metastases, we measured: CT-derived subcutaneous and visceral adipose tissue volumes, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), fasting serum adiponectin, leptin and fetuin-A. We estimated relative IHF using CS-MR and developed prediction models for IHF using a factor-clustered approach.

Results: The multivariate linear regression models showed that D-IHF was best predicted by HOMA-IR (Beta coefficient$_{\text{per doubling}}$: 2.410, 95% CI: 1.093, 5.313) and adiponectin ($\beta_{\text{per doubling}}$: 0.197, 95% CI: 0.058, 0.667), but not by anthropometrics. MR-derived IHF correlated with D-IHF (rho: 0.626; p = 0.0001), but levels of agreement deviated in upper range values (CS-MR over-estimated IHF: regression versus zero, p = 0.009); this could be adjusted for by a correction factor (CF: 0.7816).

Conclusions: Our findings show IHF is associated with measures of insulin resistance, but not measures of visceral adiposity. CS-MR over-estimated IHF in the upper range. Larger studies are indicated to test whether a correction of imaging-derived IHF estimates is valid.

KEYWORDS

Hepatic steatosis, obesity, insulin resistance, visceral adiposity, liver surgery

INTRODUCTION

Excess intra-hepatic fat (IHF), commonly termed steatosis, is present in approximately one third of patients with colorectal liver metastases (CLM).[1-3] Moderate to severe steatosis (triglyceride content >33%[4]) and steatohepatitis (steatosis with hepatocyte ballooning, lobular inflammation and fibrosis[4]) are associated with increased post-operative morbidity (two-fold increase) and peri-operative mortality (2.8- to 10-fold increase).[5, 6] Retrospective studies have shown excess body mass index (BMI), diabetes and pre-operative chemotherapy are associated with steatosis and/or steatohepatitis in the resection specimen.[3, 5, 7]

Within the general population, visceral adipose tissue (VAT) but not subcutaneous adipose tissue (SAT), correlates with intra-heaptic fat (IHF).[8-10] Consistent with this, serum surrogate biomarkers of insulin resistance independently predict for IHF.[9-11] However, IHF is determined indirectly by non-invasive imaging modalities, either using ultrasound scans in large cohorts,[12] or in clinical practice, by pre-operative chemical-shift MR (CS-MR).[13] Magnetic resonance (MR) spectroscopy may offer greater performance characteristics but its utility is limited to the research setting.[14, 15]

To directly inform the above relationships, and pre-operative assessment, we used the opportunity in patients undergoing liver resection, to quantify IHF by digital histological assessment (D-IHF) and related this with CT-derived anthropometrics (VAT and SAT), insulin-related serum biomarkers, and IHF estimated by CS-MR.

METHODS

Patients

The study schema is outlined in **(Figure 1)**. Patients planned for resection of colorectal liver metastases (CLM) at North Manchester General Hospital, Manchester, United Kingdom, were prospectively recruited between January 2012 and June 2013; all gave informed consent. All patients underwent pre-operative CT, CT-PET and CS-MR imaging as routine clinical care; the decision to proceed to resection was made at a multidisciplinary team

meeting.[16] Individuals who were recruited but did not undergo resection were still included if an intra-operative biopsy that included background liver was performed. We excluded patients undergoing re-do resections (where the regenerated liver is likely to be unrepresentative) and patients treated with more than one line of pre-operative chemotherapy (where there may be increased risk of chemotherapy-induced liver injury and/or cancer-related weight loss). Pre-operative chemotherapy was defined as any chemotherapy administered within the six-month period preceding liver resection (the definition used in a recent National Clinical Research Network Trial).[17] Data on complications, pre-operative liver enzymes levels and length of hospital were collected.

Height, weight (to derive BMI), waist circumference (WC) and hip circumference (to derive waist-to-hip ratio [WHR]) were measured before surgery. Waist circumference was measured at the level of the anterior superior iliac spines; hip circumference at the gluteal level.[7]

Development of digital histology quantification of intra-hepatic fat (D-IHF)

Seven digital images of routine H&E slides of the background liver were taken by a single consultant hepatobiliary pathologist (MaRa) at x10 magnification and maximum resolution. Each photograph was imported into Adobe Photoshop and converted to a *greyscale* image; the *levels* were then adjusted to increase the contrast. The image was magnified and the *magic wand* tool used to capture areas of fat deposition, leaving behind normal parenchyma. The D-IHF percentage was determined by counting the number of highlighted pixels divided by the total number using the *histogram* tool. Individual percentages were recorded and a mean value was derived for each patient from the 7 digital images. Seven slides were chosen based on a previous digital morphometry study reporting minimal intra- and inter-observer variability for 7 slides.[18]

The validity of this new 7-slide technique was tested by examining 43 images in one patient. The mean digitally quantified histological score from the 43 images was 4.30. We randomly sampled this data with replacement as samples n = 40, n = 35, n = 30….. n = 4

(see supplemental material). Each re-sampling was performed 500 times. For each number of slides, a distribution (n = 500) of means was derived, with a mean and 5th and 95th percentiles. Setting the limits of acceptable variation between +25% and -25% of the true mean (i.e. 3.23 and 5.38) the probability that the derived mean lay outside the pre-set limits was high if we examined less than 7 slides. Inter- and intra-rater variability for the 7-slide technique was minimal – Concordance Correlation Coefficients [CCC]: 0.97 and 0.98, respectively. Standard visual estimations of steatosis grade according to Kleiner-Brunt criteria[4] were also performed.

CT-derived VAT and SAT volumes

Pre-operative CT scans were analyzed using OsiriX software to determine SAT and VAT areas and volumes. To measure SAT area, a mean +/- 2 standard deviation fat attenuation value was derived by drawing regions of interest (ROIs) in the subcutaneous fat compartment at the level of the right renal hilum. A narrow fat attenuation window specific to each patient avoided areas of connective tissue, fibrosis and vessels. The edge of the peritoneal cavity was traced to leave behind only subcutaneous fat and the *Grow Region 2D* tool used to input upper and lower thresholds for fat attenuation. The same technique was used for VAT area, drawing an ROI in the visceral fat and subtracting the SAT. Any fat deep to the rectus sheath and outer edge of para-spinal muscles, including inter-muscular fat between quadratus lumborum and erector spinae was included in the visceral fat compartment.

To account for changes in SAT and VAT compartments at different vertebral levels[19] we derived SAT and VAT volumes. A 120mm section running cranially from the L5-S1 disc was used with the *Grow Region 3D* tool to derive volumes from individual areas. Each CT parameter was measured twice to assess intra-rater variability; measurements were repeated by an independent observer (AAP) to explore inter-rater variability (see supplemental material). There was good to excellent agreement for all measurements

(range of CCCs: 0.86-0.99). In general, inter-rater variability was greater than intra-rater variability.

Serum biomarkers of insulin resistance

Pre-surgery fasting (at least 4 hours) plasma levels of glucose and serum insulin, adiponectin, leptin and fetuin-A were determined following blood collection in standard serum tubes and centrifuged at 3,000rpm for 15min at 5℃. Serum (3-5mls) was stored at -80℃ before transfer for analysis at the Medical Re search Laboratory, University of Aarhus, Denmark. The assay techniques have been described elsewhere.[20] Briefly, glucose concentrations were analyzed by the glucose oxidase method, Beckman Instruments, CA, USA; insulin by a two-site immunospecific Enzyme-Linked ImmunoSorbent Assay (ELISA), Dako, Denmark; adiponectin by an in-house Time-Resolved ImmunoFluoroMetric Assay (TR-IFMA) based on two monoclonal antibodies and recombinant human adiponectin (R&D Systems, Abingdon, UK); leptin by a validated in-house TR-IFMA based on two commercial monoclonal antibodies and commercial recombinant human leptin as the standard, R&D Systems, Abingdon, UK. Fetuin-A levels were determined by a commercial Quantikine ELISA from R&D Systems in accordance with instructions from the manufacturer. To estimate insulin resistance, we derived a HOmeostasis Model Assessment of Insulin Resistance (HOMA-IR) value for each patient.[21] HOMA-IR has good correlation with the euglycaemic clamp (r = 0.88) and has been validated in healthy subjects.[22]

Relative hepatic fat content on chemical shift MR imaging

Relative IHF content was determined from pre-operative CS-MR scans, and cross-checked against D-IHF. We measured In- and Out- signal intensity in three 1.5-2cm^2 Regions of Interest (ROI) in liver segments II, V and VIII using a PACS (Picture Archiving and Communication System) workstation. ROI intensity was derived using the standard formula ([In-Out] / [2In]).[23] Each ROI was placed distant from major vessels, ducts and tumour

following consensus between the same two observers (EP and PM). Where it was not possible to use segments II, V and VIII, the nearest suitable segment was chosen.

Statistical analyses

All analyses were performed using Stata, version 12.1, (College Station, TX, USA). Standard approaches to categorical (chi-squared test) and continuous (Mann-Whitney U test) variables were used. Correlation matrices were constructed to assess relationships between D-IHF and other continuous variables using Spearman's Rank Correlation Coefficient. To account for multiple testing, a p-value of <0.01 was considered to indicate statistical significance.

Predictors of intra-hepatic fat content were explored using multivariable linear regression models. To reduce the right skewness of the distributions of some insulin-related biomarkers, the base 2 logarithmic transformation was used, which leads to a convenient interpretation: the beta coefficient associated with a change of one unit on the log2 scale corresponds to the slope associated with a doubling in biomarker level on the original scale. Because of the large number of variables and the anticipated high levels of correlations, we used a factor-cluster method as shown in Figure S3 (supplemental material). There were four clusters – patient-related; anthropometric measures; CT-derived anthropometrics; and insulin-related serum biomarkers. A separate model was developed for each cluster and significant ($p < 0.05$) variables selected for the final model. Model fit was assessed using the Akaike Information Criterion (AIC), Stata command: `fitstat`.[24] AIC takes in to account degrees of freedom – a lower value indicates a better fit.

Using a 1,000-patient replacement simulation model, predicted values for median digital fat percentage of 10% and BMI of 25 kg/m^2 (standard deviation: 5 kg/m^2) in a hypothetical cohort, it was estimated that 30 patients would be required to detect a significant ($p < 0.05$) Spearman correlation (probability = 96%, CIs: 79-100%).

RESULTS

Baseline characteristics

Thirty-seven patients were recruited but following exploratory laparotomy: 4 patients did not proceed to resection, of which open biopsies were taken in two. Thus, there were 35 patients eligible for D-IHF quantification.(median age 64 years, 20 males, 15 treated with pre-operative chemotherapy, see **Table 1**).

Gender-specific differences were observed across anthropometric measurements, such as men having significantly higher WC (107 cm versus 103 cm, p = 0.037) and VAT volume (1833 cm^3 versus 1001 cm^3, p = 0.003). There were also differences for insulin-related biomarkers: men had lower median leptin levels (men versus women: 13.0 µg/l versus 21.8 µg/l, p = 0.020) and lower median adiponectin levels (11.8 mg/l versus 16.2 mg/l, p = 0.013).

The median D-IHF percentage was 1.13% (range: 0% to 9.78%). Independent visual estimations of histology grade by the pathologist classified 8 cases as normal, 11 as mild steatosis, 5 cases as moderate, 1 as mild steatosis with sinusoidal congestion, 2 as sinusoidal congestion and 8 as non-specific inflammation/hepatitis. The median fat percentage for the 5 cases of visually estimated moderate grade steatosis was 6.89% (range: 4.65% to 9.09%). There was no difference in liver fat percentage by gender, diabetes status and whether or not pre-operative chemotherapy was administered. There was no association between D-IHF and post-operative complications (grade 3 and 4), liver function tests or length of hospital stay.

Correlation analysis

Results for the correlation analysis are displayed in **Table 2**. In general, pre-operative anthropometrics were strongly correlated with each other (for example, BMI and WC, rho: 0.786) and with the CT measurements (SAT volume and BMI, rho: 0.752). HOMA-IR was correlated with general anthropometrics (all rho > 0.5) but relationships with individual visceral fat compartments were weaker (for example, VAT volume and HOMA-IR, rho:

0.384). Leptin correlated with BMI, HOMA-IR, and measures of subcutaneous fat, but not visceral fat; adiponectin levels were inversely related to measures of body fat. WC and VAT volume were moderately correlated with D-IHF (rho: 0.349, p = 0.040; and 0.359, p = 0.034, respectively).

Predicting intra-hepatic fat content

Multivariate linear regression models were built with D-IHF as the dependent factor and the following factor clusters: (i) patient-related factors; (ii) anthropometrics; (iii) CT-derived measures of adiposity; and (iv) insulin-related serum biomarkers. Using a stepwise approach, we selected candidates for the preliminary model, and ultimately the final model, assessing model fit with AIC values at each step. Age and gender were included in all models. The final model, containing age, gender, weekly alcohol consumption, HOMA-IR and adiponectin is shown in **Table 3**. The multivariate linear regression models showed that D-IHF was best predicted by HOMA-IR (Beta coefficient$_{\text{per doubling}}$: 2.41, 95% CI: 1.093, 5.313) and adiponectin ($\beta_{\text{per doubling}}$: 0.197, 95% CI: 0.058, 0.667), but not by anthropometric measures of visceral adiposity.

Level of agreement between MR relative IHF and D-IHF

We tested the relation between In-Out intensity (signal fat fraction) and D-IHF. There was a moderate correlation between the In-Out phase ratio on pre-operative CS-MR and D-IHF (rho: 0.626, *P* = 0.0001) **(Figure 2a)**. Levels of agreement were explored using Bland-Altman plots. There was a trend for MR-derived relative IHF to overestimate IHF content relative to D-IHF, especially in upper range values (regression versus zero, p = 0.009) **(Figure 2b)**. Restricting the regression to a (0,0) intercept (i.e. no constant), the correction factor (CF) was 0.7816. This predicted model for CS-MR values gives a regression coefficient with D-IHF of 0.999.

DISCUSSION

Main findings

This study uniquely quantified IHF directly based on a reproducible digital histology quantification method using images from multiple sections through the liver. Our findings support the hypothesis that IHF is strongly associated with measures of insulin resistance, such as HOMA-IR and adiponectin (inversely), but not anthropometric measures of visceral adiposity. We additionally demonstrated that MR chemical-shift relative quantification may overestimate IHF content, but this can be corrected for. Larger studies are indicated to test whether a correction of imaging-derived IHF estimates are valid.

Comparison with published literature

Gender-specific differences for anthropometrics and serum biomarkers of insulin resistance (for example, increased VAT in men), have been reported previously within the metabolic literature,[8, 9, 25] as have the relationships we observed between individual anthropometrics, CT-measurements and serum biomarkers (for example, leptin correlating with subcutaneous but not visceral fat).[11, 26] The principle finding in this study – that IHF is significantly associated with measures of insulin resistance rather than physical measures of body and visceral fat – has also been described previously, but only where IHF content was quantified indirectly using MR spectroscopy.[9, 27]

Previous studies exploring predictors of excess IHF in liver resection cohorts were retrospective and relied upon data captured as part of routine clinical assessment and visual assessment of liver histology, however, both excess BMI and diabetes were consistent predictors of steatosis and/or steatohepatitis.[7, 28] Our finding that the median digital intra-hepatic fat percentage was 6.89% for the 5 cases graded as “moderate steatosis” by the pathologist (triglyceride content >33% by Kleiner-Brunt criteria[4]) is consistent with those of previous studies reporting visual estimations of steatosis grade or percentage to be at least 4 times higher than digital histological or biochemical measurements.[18, 29]

Clinical implications

It appears from this study that steatosis and steatohepatitis in liver surgery patients share aetiologies common to those seen in non-alcoholic fatty liver disease (NAFLD). Therefore, strategies that reduce IHF in NAFLD may be effective in surgical patients as well. For example, significant reductions in hepatic triglyceride content (in excess of 50%) can be achieved within a few days of starting a low-carbohydrate diet.[30, 31] Thus, using MR imaging to indirectly quantify IHF content, it may be possible to identify "high risk" patients pre-operatively and initiate a low-carbohydrate diet to reduce the chances of post-resection mortality. The next platform of studies should test the reliability and reproducibility of intra-hepatic fat measurement in CLM patients using non-invasive imaging and quantify reductions in triglyceride content following dietary intervention.

Strengths and limitations

There are several strengths to this study. First, the data was collected prospectively before liver resection surgery. Previous studies have been retrospective[5, 7, 28] limiting variables examined to those routinely captured within an institution's database, such as BMI. Second, this was a homogenous patient group because we excluded re-do resections (to avoid sampling a regenerated liver not exposed to a lifetime of risk factors for steatosis) and patients treated with multiple lines of pre-operative chemotherapy (where there may be increased risk of chemotherapy-induced liver injury and/or cancer-related weight loss). Third, we developed a reliable and reproducible method of intra-hepatic fat quantification; previous studies[5-7, 28] have relied upon visual estimations of steatosis grade or percentage, which are not reproducible[32] and overestimate the fat percentage.[18, 29] Fourth, we explored for novel predictors such as VAT volume, HOMA-IR, adiponectin and fetuin-A – parameters not previously examined in the surgical population.

A limitation of this study is the relatively small sample size. However, we attempted to compensate for this by characterizing a deep phenotype for each patient, using multiple measures of adiposity and insulin resistance. A further limitation is the cross-sectional

design, with the denominator (D-IHF) captured at the time of liver resection surgery only. Measuring liver fat content at different time points during treatment and exploring how it may alter in relation to changes in body fat distribution and/or insulin resistance would have been informative, however, findings from this exploratory work set a platform for the next generation of studies.

Future research

There are two key unanswered questions. First, is it feasible to identify patients with moderate/severe steatosis or steatohepatitis before surgery and reduce intra-hepatic fat content? And second, if it is, will this translate into improved peri-operative outcome? In the study by Vauthey *et al* [5] reporting an increase in peri-operative mortality for steatohepatitis, the interval between finishing chemotherapy and liver resection was less than 6 weeks. In subsequent studies, the chemotherapy to surgery interval has been longer,[7, 28] which may reflect a wider change in practice across other hepatobiliary units. Given the projected increase in the numbers of patients eligible for resection of CLM, and a parallel increase in the prevalence of steatosis in Western populations, better understanding of this topic is likely to benefit many patients.

ACKNOWLEDGMENTS

We wish to thank Debbie Clark and Clare Rynn from the Department of Hepatobiliary Surgery and Andrew Mackillop from the Critical Care Unit at North Manchester General Hospital, and Anne Murtagh from the Department of Medical Illustration and Margaret Roberts from the Department of Endocrinology at The Christie Hospital NHS Foundation Trust.

CONTRIBUTORS

Study concept and design: EP, DAO, PM, DJS, AGR; acquisition of data: EP, AAP, PM, MR, PC, JF; analysis and interpretation of data: EP, DAO, PM, AGR; drafting of manuscript: EP,

DAO, AGR; statistical analyses: EP, AGR; critical revision of manuscript for important intellectual content: all authors.

COMPETING INTERESTS

AGR has received honoraria and advisory board consultant fees from Novo Nordisk, manufacturer of several insulin analogues.

ROLE OF THE FUNDING SOURCE

This study was funded by HALT (Help Against Liver Tumours), registered UK charity number 1054556; and by a pump-priming grant from the Royal College of Surgeons of England.

ETHICS

Ethical approval was granted by the National Regional Ethics Service Committee North West, Greater Manchester East (11/NW/0792).

REFERENCES

1. Hamady ZZ, Rees M, Welsh FK, et al. Fatty liver disease as a predictor of local recurrence following resection of colorectal liver metastases. Br J Surg 2013;100:820-6.

2. Parkin E, O'Reilly DA, Adam R, et al. The effect of hepatic steatosis on survival following resection of colorectal liver metastases in patients without preoperative chemotherapy. HPB (Oxford) 2013;15:463-72.

3. Parkin E, O'Reilly DA, Adam R, et al. Equivalent survival in patients with and without steatosis undergoing resection for colorectal liver metastases following pre-operative chemotherapy. Eur J Surg Oncol 2014.

4. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-21.

5. Vauthey JN, Pawlik TM, Ribero D, et al. Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. J Clin Oncol 2006;24:2065-72.

6. de Meijer VE, Kalish BT, Puder M, et al. Systematic review and meta-analysis of steatosis as a risk factor in major hepatic resection. Br J Surg 2010;97:1331-9.

7. Wolf PS, Park JO, Bao F, et al. Preoperative chemotherapy and the risk of hepatotoxicity and morbidity after liver resection for metastatic colorectal cancer: a single institution experience. J Am Coll Surg 2013;216:41-9.

8. Kotronen A, Yki-Jarvinen H, Sevastianova K, et al. Comparison of the relative contributions of intra-abdominal and liver fat to components of the metabolic syndrome. Obesity (Silver Spring) 2011;19:23-8.

9. Kantartzis K, Machann J, Schick F, et al. The impact of liver fat vs visceral fat in determining categories of prediabetes. Diabetologia 2010;53:882-9.

10. Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. Gastroenterology 2012;142:711-725 e6.

11. Turer AT, Browning JD, Ayers CR, et al. Adiponectin as an independent predictor of the presence and degree of hepatic steatosis in the Dallas Heart Study. J Clin Endocrinol Metab 2012;97:E982-6.

12. Lazo M, Hernaez R, Eberhardt MS, et al. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988-1994. Am J Epidemiol 2013;178:38-45.

13. Mehta SR, Thomas EL, Bell JD, et al. Non-invasive means of measuring hepatic fat content. World J Gastroenterol 2008;14:3476-83.

14. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology 2010;51:679-89.

15. Machann J, Stefan N, Schick F. (1)H MR spectroscopy of skeletal muscle, liver and bone marrow. Eur J Radiol 2008;67:275-84.

16. Bonanni L, De'liguori Carino N, Deshpande R, et al. A comparison of diagnostic imaging modalities for colorectal liver metastases. Eur J Surg Oncol 2014;40:545-50.

17. Primrose J, Falk S, Finch-Jones M, et al. Systemic chemotherapy with or without cetuximab in patients with resectable colorectal liver metastasis: the New EPOC randomised controlled trial. Lancet Oncol 2014;15:601-11.

18. Li M, Song J, Mirkov S, et al. Comparing morphometric, biochemical, and visual measurements of macrovesicular steatosis of liver. Hum Pathol 2011;42:356-60.

19. Maurovich-Horvat P, Massaro J, Fox CS, et al. Comparison of anthropometric, area- and volume-based assessment of abdominal subcutaneous and visceral adipose tissue volumes using multi-detector computed tomography. Int J Obes (Lond) 2007;31:500-6.

20. Thomsen KL, Sandahl TD, Holland-Fischer P, et al. Changes in adipokines after transjugular intrahepatic porto-systemic shunt indicate an anabolic shift in metabolism. Clin Nutr 2012;31:940-5.

21. National Institute for Health and Clinical Excellence: Quality standard for colorectal cancer (QS20), 2012.

22. Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-9.

23. Raptis DA, Fischer MA, Graf R, et al. MRI: the new reference standard in quantifying hepatic steatosis? Gut 2012;61:117-27.

24. Jann J, Long JS. Tabulating SPost results using estout and esttab. *The Stata Journal* (2010) 10, Number 1, pp. 46–60.

25. Fox CS, Massaro JM, Hoffmann U, et al. Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. Circulation 2007;116:39-48.

26. Neeland IJ, Ayers CR, Rohatgi AK, et al. Associations of visceral and abdominal subcutaneous adipose tissue with markers of cardiac and metabolic risk in obese adults. Obesity (Silver Spring) 2012.

27. Fabbrini E, Magkos F, Mohammed BS, et al. Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. Proc Natl Acad Sci U S A 2009;106:15430-5.

28. Ryan P, Nanji S, Pollett A, et al. Chemotherapy-induced liver injury in metastatic colorectal cancer: semiquantitative histologic analysis of 334 resected liver specimens shows that vascular injury but not steatohepatitis is associated with preoperative chemotherapy. Am J Surg Pathol 2010;34:784-91.

29. Rawlins SR, El-Zammar O, Zinkievich JM, et al. Digital quantification is more precise than traditional semiquantitation of hepatic steatosis: correlation with fibrosis in 220 treatment-naive patients with chronic hepatitis C. Dig Dis Sci 2010;55:2049-57.

30. Kirk E, Reeds DN, Finck BN, et al. Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. Gastroenterology 2009;136:1552-60.

31. Browning JD, Baker JA, Rogers T, et al. Short-term weight loss and hepatic triglyceride reduction: evidence of a metabolic advantage with dietary carbohydrate restriction. Am J Clin Nutr 2011;93:1048-52.

32. El-Badry AM, Breitenstein S, Jochum W, et al. Assessment of hepatic steatosis by expert pathologists: the end of a gold standard. Ann Surg 2009;250:691-7.

LEGENDS

Figure 1. Study design. Upper panel: Pre-operative CT scans were analyzed using OsiriX software to determine subcutaneous (SAT) and visceral adipose tissue (VAT) areas and volumes (indicated by yellow arrows). Lower panel: Digital quantification with Adobe Photoshop. Areas of fat are highlighted using the magic wand; the size of the highlighted area is recorded as a pixel count

Figure 2 (a) Scatter plot of digital fat percentage (loge scale) versus In-Out signal intensity ratio on the clinical pre-operative MR scans for the 35 patients with available histology. (b) Bland-Altman plots of D-IHF versus relative IHF determined by chemical-shift MR imaging.

Table 1 Pre-operative characteristics, n = 35

Median age at hepatectomy (years)	64 (38-80)
Gender	
Men	20
Women	15
ASA Grade	
I	8
II	23
III-IV	4
Diabetes	
Yes	6
No	29
BMI (kg/m^2)	
Underweight (<18.5)	1
Normal weight (18.5-24.9)	9
Overweight (25.0-29.9)	16
Obese (≥30.0)	9
Site of primary tumour	
Colon	13
Rectum	22
Dukes' stage of primary tumour	
A	1
B	9
C	19
Complete pathological response	1
Synchronous resection	3
Liver first, primary in-situ	2
Pre-operative CEA level – median (IQR) (ng/ml)	5 (2 -27)
Pre-operative chemotherapy	
Yes	15 *
No	20 †
Resectable EHD present	
Yes	1
No	36
Serum biomarkers – median (IQR)	
HOMA-IR	1.0 (0.6 – 1.3)
Adiponectin (mg/l)	12.4 (10.0 – 16.2)
Leptin (ug/l)	14.8 (9.1 – 25.2)
Fetuin A (g/l)	0.46 (0.40 – 0.56)

* 6 received pre-operative liver chemotherapy; 9 finished adjuvant bowel chemotherapy <6 months before liver resection

† 15 patients never received chemotherapy; 5 finished adjuvant bowel chemotherapy ≥6 months before liver resection

ASA: American Society of Anesthesiology. BMI: Body Mass Index. CEA: CarcinoEmbryonic Antigen. EHD: Extra-hepatic disease. IQR: inter-quartile range.

Table 2 Correlations (Spearman coefficients) of various anthropometric measures, serum markers and intra-hepatic fat (%)

	BMI	WC	SAT Vol	VAT Vol	HOMA-IR	Leptin	Adiponectin	Fetuin
WC	0.79*							
SAT Vol	0.75*	0.75*						
VAT Vol	0.65*	0.76*	0.53*					
HOMA-IR	0.56*	0.57*	0.39*	0.38*				
Leptin	0.46*	0.18	0.46*	0.08	0.38*			
Adiponectin	-0.17	-0.33*	-0.10	-0.37*	-0.13	0.00		
Fetuin-A	0.13	-0.03	-0.01	0.06	-0.14	0.16	-0.02	
Intra-hepatic fat %	0.28	0.29	0.25	0.36*	0.29	0.14	-0.33	0.15

*p value < 0.01

Table 3 Multivariable linear regression model with D-IHF as the dependent variable

Variable	Beta coefficient	95% CIs	P value	Model fit (AIC)
Preliminary model (after factor cluster analyses)				3.820
Age (per year increase)	0.0343	-0.0221, 0.9074	0.222	
Gender (men versus women)	1.5857	-0.6118, 3.227	0.058	
Alcohol (≥ 10 units/week versus < 10 units/week)	1.5573	0.1677, 2.9469	0.030	
BMI (per 1 kg/m^2 increase)	0.0330	-0.2153, 0.2812	0.787	
Measured WC (per 1 cm increase)	-0.0032	-0.1215, 0.1151	0.956	
CT-derived WC (per 1 cm increase)	-0.0085	-0.1258, 0.1088	0.882	
VAT volume (per 1 cm^3 increase)	0.0003	-0.0009, 0.002	0.575	
HOMA-IR (per doubling)	2.0215	0.7491, 5.4551	0.157	
Adiponectin (per doubling)	0.2121	0.0566, 0.7940	0.023	
Final model				3.623
Age (per year increase)	0.0339	-0.0173, 0.0850	0.186	
Gender (male versus female)	1.3688	0.0879, 2.650	0.037	
Alcohol (≥ 10 units/week versus < 10 units/week)	1.7596	0.5559, 2.9633	0.006	
HOMA-IR (per doubling)	2.4137	1.0934, 5.3134	0.030	
Adiponectin (per doubling)	0.1970	0.0582, 0.6672	0.011	

AIC: Akaike Information Criterion

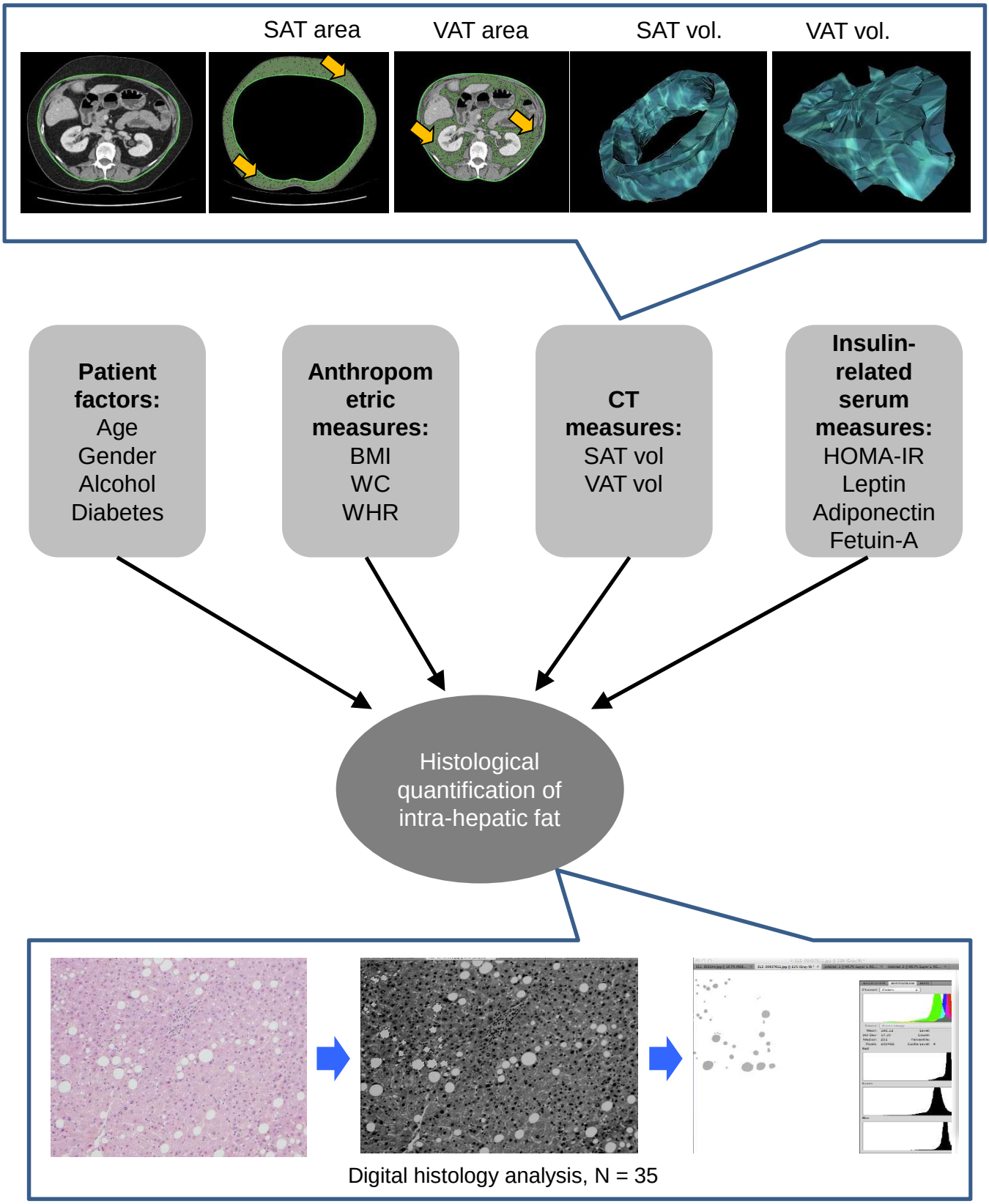

SAT area
VAT area
SAT vol.
VAT vol.
Patient factors:
Age
Gender
Alcohol
Diabetes
Anthropometric measures:
BMI
WC
WHR
CT measures:
SAT vol
VAT vol
Insulin-related serum measures:
HOMA-IR
Leptin
Adiponectin
Fetuin-A
Histological quantification of intra-hepatic fat
Digital histology analysis, N = 35

Figure 1

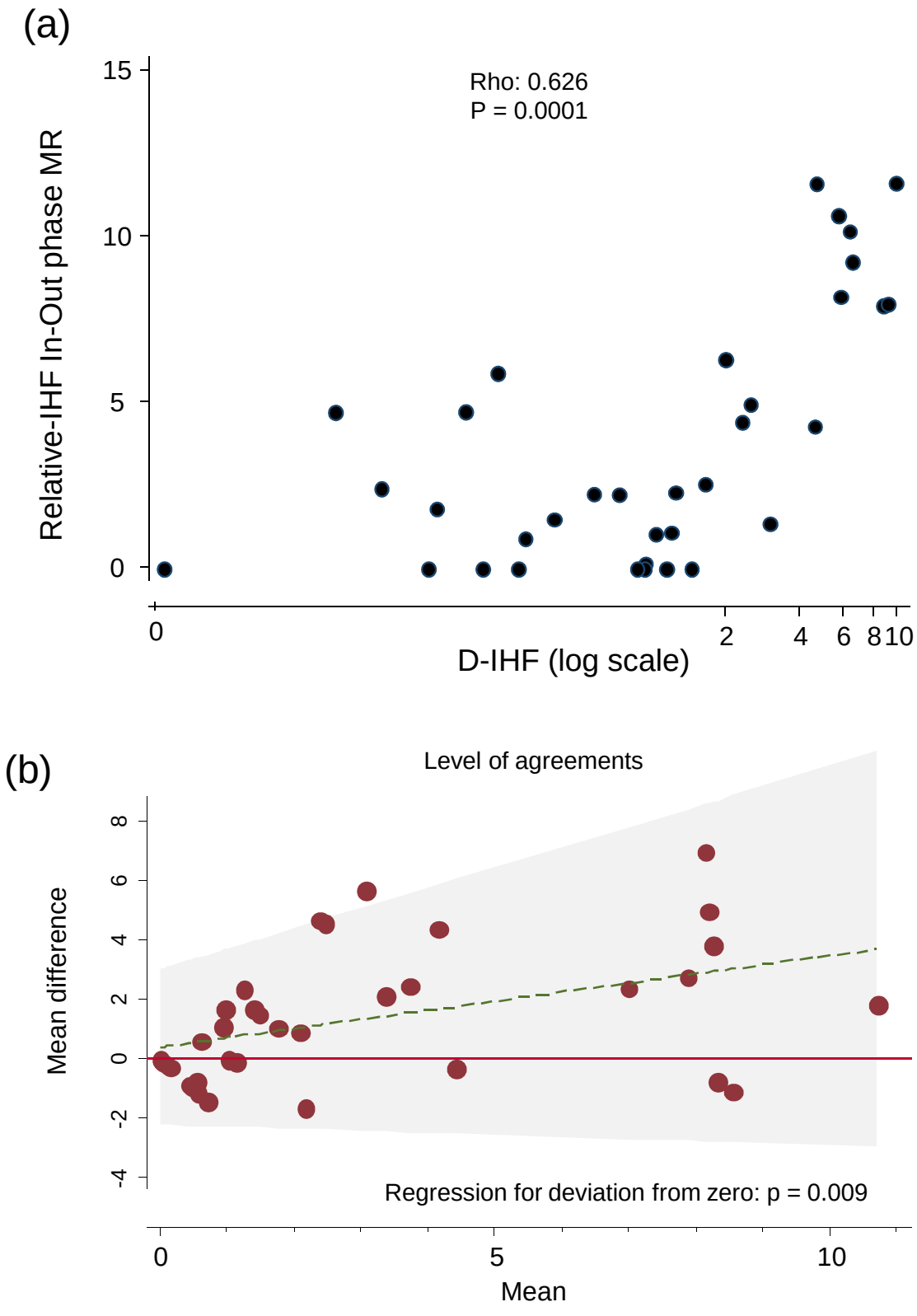

(a)
Rho: 0.626
P = 0.0001
Relative-IHF In-Out phase MR
15
10
5
0
0
2
4
6
8
10
D-IHF (log scale)
(b)
Level of agreements
Mean difference
8
6
4
2
0
-2
-4
Regression for deviation from zero: p = 0.009
0
5
10
Mean

Figure 2

SUPPLEMENTARY MATERIAL

Digital Histology Quantification of Intra-hepatic Fat in Patients undergoing Liver Resection

Parkin et al.

SUPPLEMENTAL METHODS

Validity of the 7-slide technique

Li et al.[1] examined 7 histological sections and scored intra-hepatic fat content as the mean of these 7 scores. Based on this technique, and for pragmatic reasons, we examined 7 slides as well. To test the validity of this technique, in one patient with an average amount of fat on 7 slides, I examined 43 images. The mean digitally quantified histological score from the 43 was 4.30. This was taken as the *true* mean. I then randomly sampled this data with replacement as samples n = 40, n = 35, n = 30 …….. n = 4 **(Figure S1)**. Each re-sampling was performed 500 times for each theoretical number of slides. For each number of slides, a distribution (n = 500) of means was derived, which itself had a mean and 5th and 95th percentiles. If we set the limits of acceptable variation between +25% and -25% of the true mean (i.e. 3.23 and 5.38) the probability that the derived mean lies outside the pre-set limits is high if we examined less than 7 slides. The 7-slide technique was therefore accepted as 'fit for purpose'.

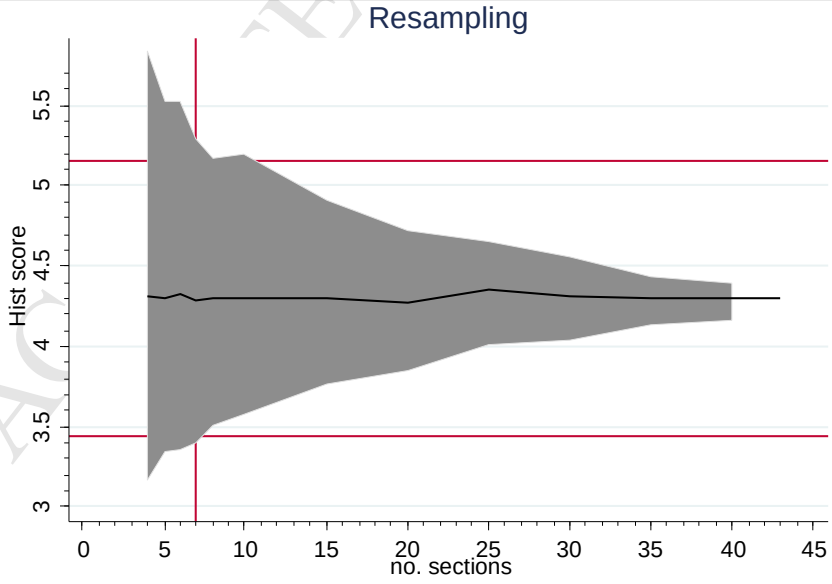


Figure S1 The distribution (n = 500) of means was derived, which has a mean (thick black line) and 5th and 95th percentiles (shaded area). If the limits of acceptable variation are between +25% and -25% of the true mean (3.23 to 5.38, red dashed lines), the probability that the derived mean lies outside these limits is high if we examine less than 7 slides.

To assess the reproducibility of the Adobe 7-slide technique, the variability in fat quantification according to (i) the same rater at different time points (intra-rater variability) and by (ii) different raters (inter-rater variability) was assessed using the concordance correlation coefficient (Stata command: `concord`). Histology measurements were repeated at separate sittings, for the first 19 patients to assess intra-rater variability. A second rater independently analysed histology 7 slides for every fifth patient to assess inter-rater variability for the technique. Both correlations were excellent: the concordance correlation coefficient for intra-rater variability was 0.98; for inter-rater variability it was 0.97.

Intra- and inter-rater variability

Each CT parameter was measured twice at separate sittings to explore intra-rater variability. A radiologist (Dr Andrew Plumb) performed measurements independently to explore inter-rater variability. The concordance correlation coefficient was used for the statistical analysis. Results of inter- and intra-rater variability for the 37 patients ultimately recruited in to the OSCAR study is shown in **Table S1**. There was good to excellent agreement for all measurements. In general, WC and SAT and VAT volumes showed the strongest correlations, and inter-rater variability was greater than intra-rater variability.

Table S1 Inter- and intra-rater variability for CT fat measurements

Measurement	Inter-rater variability (CCC, rho)	Intra-rater variability (CCC, rho)
WC	0.991	0.997
SAT thickness	0.922	0.978
VAT thickness	0.862	0.895
SAT area	0.906	0.925
VAT area	0.956	0.975
SAT volume	0.965	0.991
VAT volume	0.971	0.986

CCC: concordance coefficient

Scatter plots to show inter-rater variability and levels of agreement for the three different VAT measurements (thickness, area and volume) are shown in **Figure S2**.

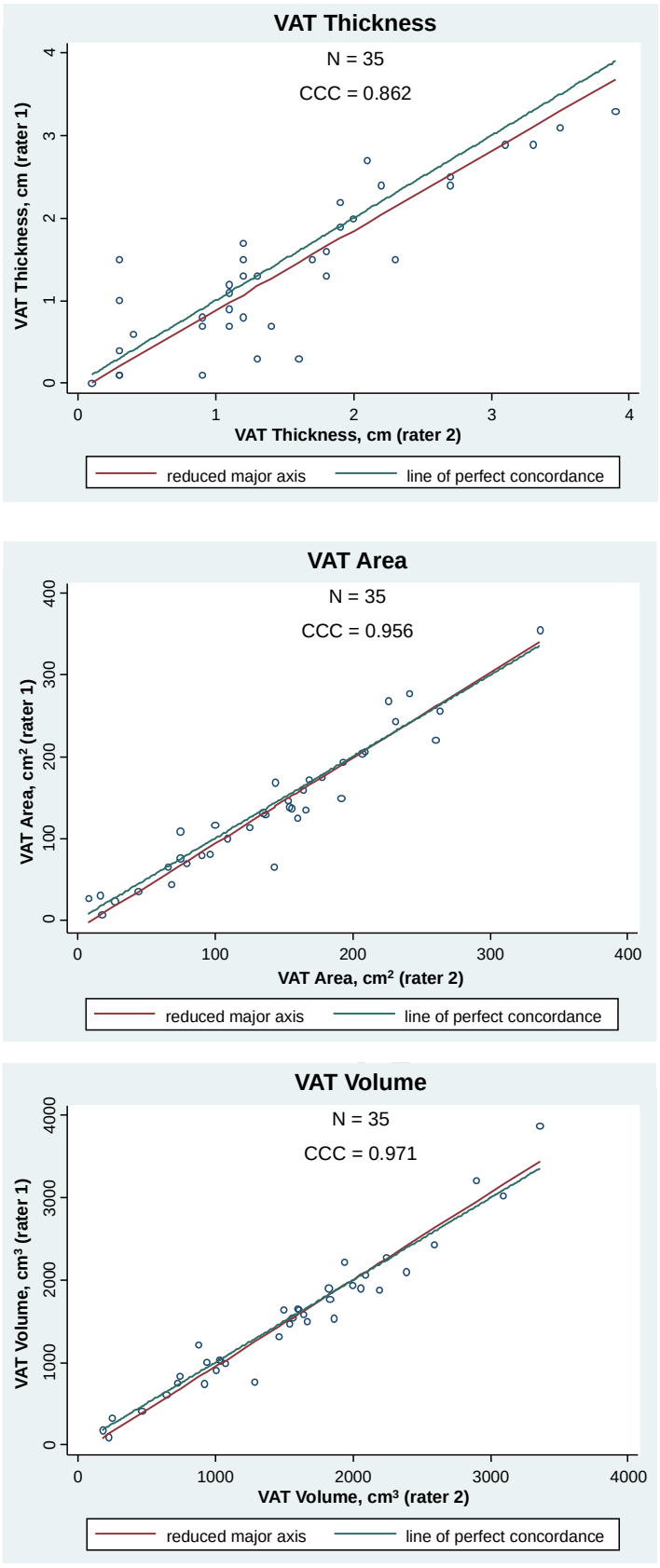


Figure S2:Inter-rater variability and levels of agreement for the three CT measures of visceral fat – thickness, area and volume

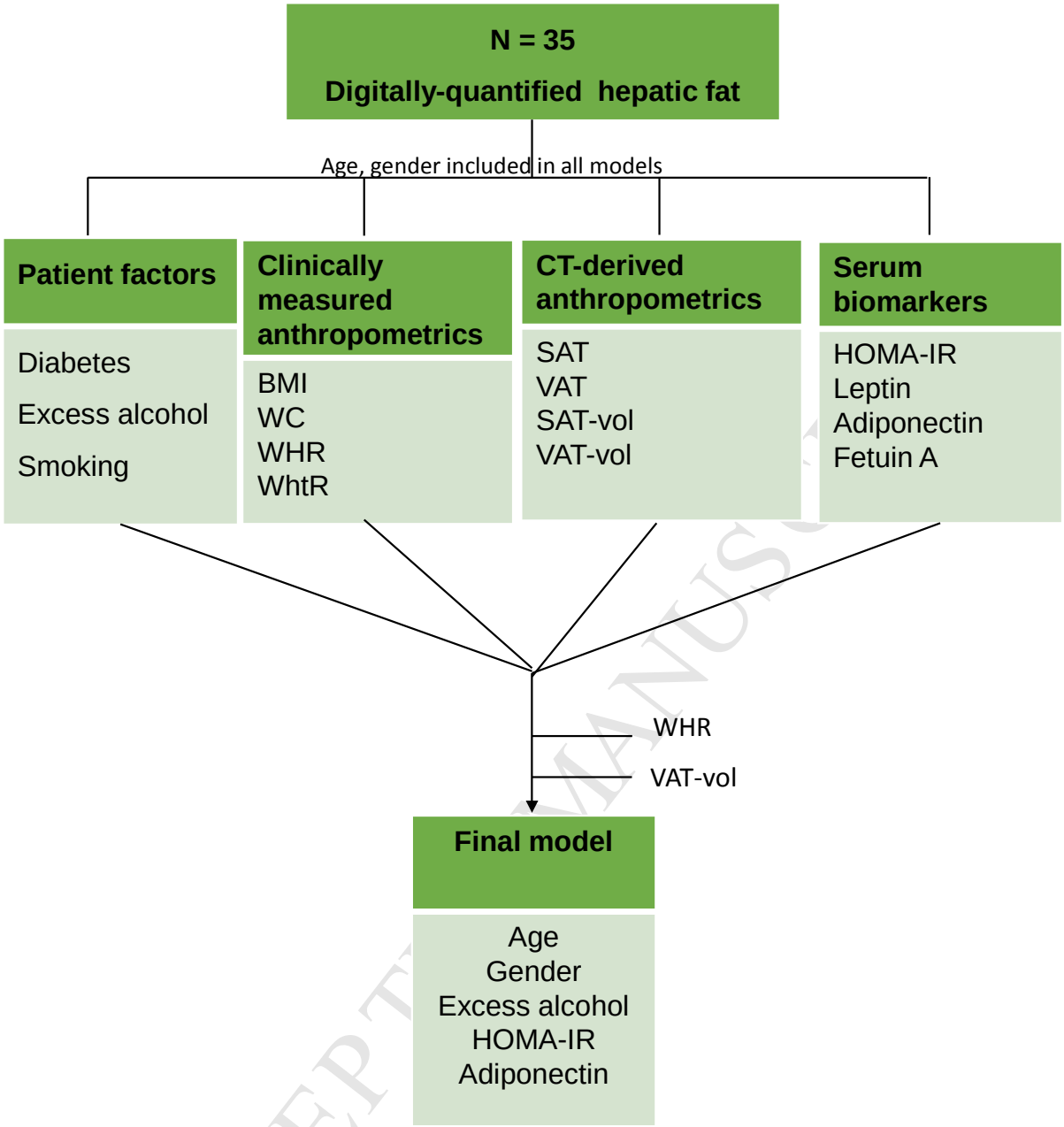


Figure S3: Factor-cluster approach to multivariate linear regression model

References to Supplemental Material

1. Li M, Song J, Mirkov S, Xiao SY, Hart J, Liu W. Comparing morphometric, biochemical, and visual measurements of macrovesicular steatosis of liver. *Hum Pathol* 2011; **42**(3)**:** 356-60.