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HIGHLIGHTS
Step-by-step description of intraorgan fat quantification in liver and pancreas
Description of in vivo measurement of hepatic lipoprotein triglyceride export
Application of novel magnetic resonance and lipoprotein turnover methods
Easy to reproduce methods without use of isotopes or ionization radiation

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Protocol
Measurement of intraorgan fat and hepatic output of triglycerides in human type 2 diabetes by magnetic resonance and intralipid infusion techniques

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SUMMARY
Liver fat content and the linked rate of export of triglyceride are central to the etiology of type 2 diabetes, as well as to the cardiovascular effects of fatty liver disease. Measurement in humans of intrahepatic and intrapancreatic fat content is described using magnetic resonance techniques and quantification of the rate of hepatic secretion of very low density lipoprotein using a non-isotopic competitive blocking of tissue uptake. This protocol is non-invasive, can be repeated sequentially, and does not involve ionizing radiation.

For complete details on the use and execution of this protocol, please refer to (Taylor et al., 2018) and (Al-Mrabeh et al., 2020b).

BEFORE YOU BEGIN
Overview
In this protocol, step-by-step procedures to assess hepatic lipid metabolism and export will be explained. Magnetic resonance imaging (MRI) is used to quantify fat content within the liver and pancreas. Acquisition of raw data, data analysis to derive fat fraction image, and measurement of fat percentage were described. Also described are the detailed clinical and laboratory procedures to measure the rate of triglyceride output by the liver. The latter is achieved using a 75-min intralipid infusion, thus blocking clearance of very low density lipoprotein triglycerides (VLDL-TG) and permitting the time-dependent accumulation of newly synthesized VLDL-TG by the liver.

Ethics approval
Participants were part of the mechanistic studies of the Diabetes Remission Clinical Trial (DIRECT/ISRCTN03267836) (Taylor et al., 2018;Al-Mrabeh et al., 2020a;Al-Mrabeh et al., 2020b). Ethical approval was obtained from the West of Scotland and North Tyneside Ethics Committees, and written consent was obtained from all participants.

MR acquisition for intraorgan fat content

© Timing: 15–30 min

The method is based on a modified magnetic resonance 3-point Dixon acquisition (Glover and Schneider, 1991) for separation of fat-water signals. We first used it to quantify fat content within the liver and the pancreas (Lim et al., 2011;Steven et al., 2016a;Steven et al., 2016b). The method was further refined for more precise measurement of intrapancreatic fat content.
1. Before data acquisition, access to an MRI scanner is required. We used a 3.0T Philips Achieva scanner (Best, The Netherlands), but see alternatives section.
   a. Scanner should be equipped with a receive coil array with at least six channels.
   b. The scanner should be programmed to acquire 3-point Dixon and balanced turbo field echo (BTFE) sequences (also known as true-FISP sequences on some scanners). The 3-point Dixon scans can be set up and processed by experienced magnetic resonance physicists or commercial packages are available from MR vendors.
2. After data acquisition, a PC with Intel Core I7 or above (Dell, USA) is required with the following software:
   a. MATLAB if physicists are processing fat fraction maps (MathWorks, USA).
   b. ImageJ (National Institutes of Health, USA).

△CRITICAL: Contraindications to MR scanning (or metallic implants) must be ascertained in advance and verified by a qualified radiographer. This includes cardiac pacemakers, aneurysm clips, certain metal implants, and claustrophobia. Unless a wide-bore scanner is used, it is not generally feasible to study individuals with BMI > 45 kg/m².

Measurement of hepatic lipoprotein-TG production

🎯 Timing: 30–45 min

This method is based on blocking tissue uptake of very low density lipoprotein triglycerides (VLDL1-TG) by intravenous intralipid infusion to study lipoprotein kinetics (Al-Shayji et al., 2007).

The method is based on competition between chylomicrons and larger VLDL particles (VLDL1) in binding to lipoprotein lipase (LPL). The intralipid emulsion contains chylomicron-like lipoprotein which, because of similar LPL binding characteristics, causes accumulation in plasma of newly released VLDL1-TG. Ultracentrifugation and density gradient techniques are used afterwards to separate the VLDL1 fraction from sequential blood during the infusion period (75 min). The rate of VLDL1-TG export can thus be derived. In our weight loss studies, all medications were terminated on the day before study, as this protocol may be affected by medications that alter lipid or carbohydrate metabolism. The only contra-indication to the test is presence of gross hypertriglyceridemia. No medications are known to interfere with the measurement.

Clinical procedures

🎯 Timing: 10–15 min

3. Human subject after overnight fasting (10–12 h), well hydrated (unrestricted drinking of water) and having avoided excessive physical activity in the previous 24 h. Taxi transport is normally provided if required to minimize the effect of stress and physical activity on the metabolic rates.
4. Measure weight and calculate intralipid bolus (0.1 g/kg body mass): 20% intralipid bolus volume (mL) = 0.5 x weight (kg).
5. Draw up bolus in syringe and label “20% intralipid bolus.”
6. Calculate 10% intralipid infusion rate at the optimal rate of (0.1 g/kg/h).
7. Label 6 x EDTA tubes (10 mL K2E BD Vacutainer) as −10, +0, +15, +45, +60, and +75 min in a sampling tray.
8. Label 6 x 5 mL graduated plastic tubes with the subject ID and by time points for collecting plasma.
9. Set up the infusion pump (Arcomedical Infusion).
10. Benchtop centrifuge (Harrier 18/80R centrifuge) cooled to 4°C.
11. Prepare box of ice (4°C) to hold cooled samples.

Separation of chylomicrons and intralipid from plasma by low speed centrifugation

@ Timing: 10–15 min

12. Prepare 1.006 g/mL density solution (Table 1).
13. Set up the six-channel peristaltic pump (Joyfay International, USA).
14. Get the Sigma 6K15 centrifuge with 12170 rotor ready.
15. Label 6 315 mL Falcon tubes (−10, +0, +15, +45, +60, and +75 min) and place in a rack.

Separation of VLDL1 lipoprotein fraction by ultracentrifugation

@ Timing: 10–15 min

16. Prepare density solutions as described in Table 1.
17. Book access to the L7-80 ultracentrifuge (Beckman Coulter, USA) and the SW40Ti Rotor.
18. Ready the six-channel peristaltic pump for dispensing.
19. Place 6× ultracentrifuge tubes (SETON SCIENTIFIC, USA) in rack and label as −10, +0, +15, +45, +60, and +75.
20. Prepare glass Pasteur pipettes by drawing a 8 cm fine tip using flame. Get Pyrex 5 mL cylinder for measuring lipid.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | Sigma-Aldrich, UK | Cat No: S9888 |
| Sodium chloride (NaCl) | Alfa Aesar, USA | Cat No: 14037 |
| Sodium bromide (NaBr) | VWR International, UK | Cat No: 102525P |
| Sodium hydroxide (NaOH) | VWR International, UK | Cat No: 100935V |
| Na₂ EDTA | VWR International, UK | Freeflex |
| Sodium chloride (0.9%) | Fresenius Kabi, UK | Intralipid 20% |
| Intralipid 20% | Fresenius Kabi, UK | Intralipid 20% |
| (Continued on next page) | | |
MATERIALS AND EQUIPMENT

⚠ CRITICAL: All intralipid infusion and blood sampling procedures should be carried by a medically qualified doctor or a research nurse.

Alternatives: (i) Use of other centrifuges or pumps for infusion of intralipid or layering of density solutions is possible. (ii) The protocol uses a 3T whole body magnetic resonance scanner (Philips, Best, The Netherlands) for MR data acquisition, although a 1.5 T scanner would also be suitable for data acquisition. We used six-channel cardiac array (Philips), other abdominal coils with similar or more channels could also be used. The 3-point Dixon acquisition and processing was implemented by an experienced magnetic resonance physicist: commercial packages to produce fat fraction mapping of the abdomen are now also available.

STEP-BY-STEP METHOD DETAILS

Magnetic resonance for intraorgan fat content: Programming of scanner

⏱ Timing: 1 h

These procedures will allow acquisition of separate MRI axial scans of the abdomen for measuring liver and intrapancreatic fat content (3-point Dixon). The Dixon acquisition contains three gradient-echo scans with adjacent out-of-phase and in-phase echoes (time to repetition/time to echo/averages/flip angle=50 ms/3.45, 4.60, 5.75 ms/1/5°, bandwidth 435 Hz/pixel). Field of view was set according to patient size (400–480 × 300 mm), and zero filled to give a resolution of 1.39 × 1.40 mm. 5 mm thick sections were used to cover the pancreas during breath holding. Liver scans were acquired during breath holding to cover the liver with slice thickness 10 mm. A balanced turbo field echo (BTFE) image with 5 mm sections matched to the Dixon scan was acquired during...
breath holding, to distinguish high signal intensity from vessels with visceral fat with lower intensity signals from the pancreas. BTFE images contain a mixed T1/T2 contrast. This is used to delineate the boundaries of the pancreas from adjacent structures, including the surrounding visceral fat, the splenic vein, the superior mesenteric vessels the inferior vena cava and duodenum. Repetition time/echo time/flip angle = 3.1 ms/1.6 ms/40°, turbo factor 95, parallel imaging factor 2, bandwidth 1,156 Hz per pixel). The field of view and zero filling were matched to the Dixon imaging.

BTFE sequences are readily available on modern scanners. Dixon sequences can either be programmed by magnetic resonance physicists (as in this work) or commercial packages can be used to produce fat fraction maps are available from MR vendors.

**Magnetic resonance for intraorgan fat content: MR data acquisition**

© Timing: 30–45 min

1. After consenting and checked for metal contraindications, participant changes into clothing with no metallic components.
2. Participant placed in supine position within the scanner with respiratory bellows (or equivalent to allow the radiographers to monitor breath holding) and a patient alert button in hand.
3. Earplugs and the headphones provided for cancelation of the noise inside the scanner.
4. A BTFE survey scan to ensure the coil is properly placed to cover the scan area.
5. Liver scan: The slices are prescribed based on the survey scan and subjects were asked to breath in and out three times before being asked to hold their breath. The scan can be divided into two breath holds (12–16 s each) to reduce breath hold duration.
6. Pancreas scan: The Dixon and BTFE scans are similarly prescribed and acquired during breath holding. The Dixon scan can be divided into two breath holds to reduce breath hold duration.
7. The total time in the scanner is 20–30 min, including positioning.
8. Participant is taken out of the scanner and back to the changing room with the help of radiographic assistant.
9. Raw data are copied from the scanner to a local network driver for further analysis.

**Magnetic resonance for intraorgan fat content: MR Data processing: liver scan**

© Timing: 10–15 min

If the Dixon sequence has been written by local MR physics support, then the raw data require further processing using a custom script written in MATLAB (Key resources table) to produce the fat fraction map. Where a commercial product has been used, the fat fraction map will be available directly from the scanner.

10. Create fat fraction map of the liver scan by custom MATLAB script (fatcalc_direct, see Figure S1).
11. The script prompts for the number of scans (if more than 1 breath hold was used). Scans should be selected from inferior to superior prescription.
12. Make sure that fat/water assignment were allocated correctly by the software (subcutaneous/visceral fat is white/other low fat fraction tissues are darker).
13. Change the threshold to ensure that background noise outside the subject is suppressed while the liver is visible.
14. Click Ok; an Analyze 7.5 format file is produced that can be read with ImageJ.
### Magnetic resonance for intraorgan fat content: MR data processing: pancreas scan

- **Timing**: 8–15 min

15. Dixon scan: Follow the same procedures for liver Dixon scan above
16. BTFE scan: Convert the scanner export format to Analyze 7.5 format using a custom MATLAB script (btfe_process), see Figure S4.

### Magnetic resonance for intraorgan fat content: Liver fat measurement

- **Timing**: 10–15 min

Detailed procedures are explained in Figure S2/Figure S3, and examples of fat distribution and final fat percentage are presented in two subjects (1 with type 2 diabetes and 1 control), Table 2/Figure 1.

17. Open ImageJ.
18. Open the Analyze 7.5 file containing the liver fat fraction map.
19. Select Image > Adjust > Window level to adjust brightness.
20. Select Analyze > Tools > ROI manager > Add.
21. Rename the ROI to the image slice number.
22. Select Polygon tool and draw a region of interest (ROI) to select a homogeneous area of the liver (avoid major blood vessels that appears as dark empty areas, Figure S2E). Save the slice by clicking update.
23. Repeat the step above on five representative slices (slices 1–5).
24. In the ROI manager click More > Save to save the ROI file.
25. Press measure from the ROI manager window.
26. In the Results window click File > Save As > Save as Excel file.
27. In the Excel, take the average of the five slices to get the percentage of fat within the liver.

### Magnetic resonance for intraorgan fat content: Pancreatic fat measurement

- **Timing**: 20–30 min

The MR-opsy method was used for precise quantification of intrapancreatic fat within the parenchyma of the pancreas (Al-Mrabeh et al., 2016; Al-Mrabeh et al., 2017).

### Magnetic resonance for intraorgan fat content: Pancreas fat without thresholding

- **Timing**: 10–15 min

Detailed procedures are explained in Figure S5, and examples of intrapancreatic fat percentage are presented in two subjects (1 with type 2 diabetes and 1 control), Table 3/Figure 2.

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**Table 2. Example of liver fat measurements in a person with type 2 diabetes and a non-diabetic control**

|          | Slice 1 | Slice 2 | Slice 3 | Slice 4 | Slice 5 | Average |
|----------|---------|---------|---------|---------|---------|---------|
| T2DM     | 24.8    | 23.2    | 21.2    | 20.7    | 21.4    | 22.3    |
| NDC      | 1.7     | 1.7     | 0.6     | 1.4     | 1.0     | 1.3     |

Two males aged 59 and 61 years, body weight 131.8 and 85.9 kg, with and without type 2 diabetes (T2DM/NDC), respectively, were selected. The table illustrates the expected minor variance between slices and the considerably higher liver fat levels typical of type 2 diabetes. NAFLD is defined as a liver fat content over 5.5%.
28. Open ImageJ.
29. Open the matched Analyze 7.5 files containing the pancreas fat fraction map and the BTFE image.
30. Select Image > Adjust > Window level to adjust brightness.
31. Select Analyze > Tools > ROI manager > Add.
32. Rename the ROI to the image slice number.
33. Select Oval tool of ImageJ to create a circular region of interest (100 mm²) on a representative slice of the pancreas to select a homogeneous areas on the head of the pancreas (slice 1-01). It is important to position the selection within the parenchymal tissues away from the borders to avoid contamination of surrounding visceral fat. The BTFE scan should be used to provide the boundary of the pancreas and the major blood vessels (bright color). Save the slice by clicking update.
34. Repeat the same step by copying the same selected region on the body (slice1-02), and tail (slice1-03) of the pancreas.
35. Repeat the step above on a second representative slice (slice2).
36. In the ROI manager click More > Save.
37. Select measure from the ROI manager window.
38. In the results window click File > Save As > Save as Excel file.
39. In Excel, take the average of the 6 ROIs to get the percentage of fat within the pancreas.

Magnetic resonance for intraorgan fat content: Pancreas fat with thresholding

Timing: 10–15 min

The tissues of the pancreas are very heterogeneous. The purpose of this step is to minimize the effect of tissue heterogeneity and to exclude contribution of visceral fat invasion between the lobules of the pancreas (Al-Mrabeh et al., 2017). Procedures are explained in Figure S6, and examples of intra-pancreatic fat percentage using thresholding method are presented in two subjects (1 with type 2 diabetes and 1 control), Table 3/Figure 2.

40. Do the same steps until step 35 above.
41. Highlight all ROIs of each slice (slice1-01/ slice1-02/ slice1-03) by holding simultaneously Ctrl and the shift key and right click with the mouse.
42. Then, in the ROI manager click More > OR (combine) > add > rename.
43. Name the combined 3 selections as average slice 1.
44. Go to analyze > Histogram > OK > copy.
45. Paste into an Excel spreadsheet template where thresholding is applied to exclude signals below and above specific limits (<1% and >20%).
46. The average of the two pancreas slices is used to derive the final pancreas fat percentage.

Pause point: No need to continually re-create the 100 mm² MR-opsy selection. You simply do this once and save it as template, then copy the ROI and use ImageJ tool to adjust the positions (Figures S5/S6).

△ CRITICAL: The thresholding step is optional when fat content is low within the pancreas, but it became essential with high interlobular fat infiltration (Figure S8). We recommend applying the threshold for all subjects for consistent and more precise measurements (Table 3/Figure 2).

△ CRITICAL: For more robust pancreatic fat quantitation, it is essential that the analyzer is blinded to participant’s trial status (We use a blinding scheme based on allocation of random letters, i.e., DiRECT-APL-C, Figures S4/S5/S6).

### Measurement of hepatic lipoprotein-TG production: Intralipid infusion and plasma collection

**Timing: 1 h 40 min**

This method allows quantification of hepatic production rate of VLDL1-TG by intralipid infusion. The method was validated and described in details (Al-Shayji et al., 2007).

47. After undertaking the MR scans, the participant remains in the fasting state.
48. Antecubital veins of both arms cannulated with 18G venflon and maintained patent using normal saline.
49. At −15 min prior to lipid infusion, withdraw 9 mL of blood using 10 mL syringe and transfer to the −15 labeled EDTA tube and leave at 22°C until step 54.
50. At 0 min withdraw 9 mL and transfer it to the +0 tube.
51. Injected through one cannula (within 60 s) the 20% intralipid bolus that were prepared previously.
52. Start immediately a continuous infusion of 10% intralipid as calculated previously for 75 min.
53. Withdraw 9 mL of blood at 15, 45, 60, and 75 min after infusion and transfer to the already labeled EDTA tubes.
54. Prepare plasma by centrifugation of blood using the refrigerated Harrier 18/80R centrifuge (3,000 × g, 10 min, 4°C).
55. A breakfast is provided to the participant.

Pause point: Blood samples were processed in pairs (−15/+0) or +60/+75 during centrifugation and preparation of plasma, then placed in ice (4°C).

| Slice | 1-01 | 1-02 | 1-03 | Ave. Slice 1 | 2-01 | 2-02 | 2-03 | Ave. Slice 2 | Average No Threshold % | Average with Threshold % |
|-------|------|------|------|--------------|------|------|------|------------|------------------------|------------------------|
| T2DM  | 35.6 | 28.8 | 34.2 | 32.9         | 39.4 | 41.4 | 31.3 | 37.4       | 32.9 + 37.4/2=35.2    | 14.0 + 13.3/2=13.6   |
| NDC   | 3.4  | 3.5  | 3.6  | 3.6          | 3.4  | 1.9  | 2.3  | 2.6        | 3.6 + 2.6/2=3.1      | 4.8 + 4.3/2=4.6      |

Two females aged 61 and 62 years, body weight 97.4 and 68.8 kg, with and without type 2 diabetes (NDC), respectively, were studied. A subject with Type 2 diabetes (T2DM) was selected with high intrapancreatic fat content. The expected variance between slices is also illustrated to show the effect of the thresholding.
Measurement of hepatic lipoprotein-TG production: Separation of chylomicrons and intralipid

- **Timing:** 60 min

Chylomicrons and intralipid (Svedberg flotation rate (Sf) > 400, density < 1.006 g/mL) are separated from plasma by short run (30 min) centrifugation of plasma which has been overlayed with stock solution 1 at density 1.006 g/mL (Table 1).

56. Use peristaltic pump to overlay 4 mL of 1.006 g/mL density solution on the top of the 2 mL plasma sample in 15 mL Falcon tubes.
57. Centrifuge for 30 min at 15°C (10,000 x g, brake: 5, Acceleration: 9) using the 12170 rotor of the Sigma 6K15 centrifuge.
58. Remove tubes carefully to minimize mixing of the separated two layers. Using a plastic pipette, remove the upper layer (chylomicrons and intralipid on the top). Transfer the lower 2 mL to a fresh-labeled 5 mL graduated plastic tube using a 10 mL syringe with 19G needle.
59. Samples can be processed directly at this stage for separation of VLDL1 fraction. However, samples could be saved (−40°C) to be proceeded within weeks to few months.

Measurement of hepatic lipoprotein-TG production: Separation of VLDL1 lipoprotein fraction

- **Timing:** 3–4 h

VLDL1 is isolated from plasma by a modification of the cumulative ultracentrifugation density gradient technique (Lindgren et al., 1972). Density solutions are prepared from stock solutions at density 1.006 g/mL and d 1.182 g (Table 1). The densities are measured within 0.001 g/mL using a calibrated 1,000 mL pipette and analytical balance. To save time, it is important that the frozen samples allowed to defrost in ice (4°C) at 22°C for 30–40 min prior to analysis.

60. Adjust the density of the plasma sample to 1.118 g/mL by weighing up 0.341 g NaCl and adding gradually to the tube containing 2 mL plasma with gentle mixing by inverting the tube (vortexing is not recommended to avoid foam formation).
61. Use the peristaltic pump to layer in order these solutions in the ultracentrifuge tubes (the speed of the pump should be adjusted properly to avoid disturbing the layers by starting at low speed and increase the speed gradually).
   a. 0.5 mL of Stock 2 (1.182 g/mL).
   b. 2 mL of density adjusted plasma (1.118 g/mL).
   c. 1 mL of Solution 3 (1.0988 g/mL).
   d. 1 mL of Solution 4 (1.0860 g/mL).
   e. 2 mL of Solution 5 (1.0790 g/mL).
   f. 2 mL of Solution 6 (1.0722 g/mL).
   g. 2 mL of Solution 7 (1.0641 g/mL).
   h. 2 mL of Solution 8 (1.0588 g/mL).

62. Cap the tubes carefully and attach the buckets to the SW40Ti rotor. As close as possible to the Beckman ultracentrifuge.

63. Carry the rotor with the attached tubes carefully to avoid disturbing the layers, and mount the rotor on the centrifuge.

64. Ensure that the rotor is correctly aligned with the centrifuge pins.

65. Close the door and switch on the vacuum.

66. Once the vacuum is established, start the run at 278,000 × g for 98 min at 23°C with the brake off.

67. After completion the run, switch off the vacuum, remove the rotor containing the buckets and carefully place each bucket in the stand.

68. Using a drawn glass pipette, remove the top fraction from each tube. Always ensure that the fraction from is removed the very top, by placing the pipette on the edge of the meniscus and drawing air up along with the sample.

69. Use a glass-measuring cylinder for measuring the amount of VLDL1 fraction. This will ideally be 1 mL but if more volume was taken, the final concentration should be adjusted accordingly.

**Measurement of hepatic lipoprotein-TG production: Triglycerides quantification of VLDL1 fraction**

*Timing: 15 min*

This is carried out using on a fully automated fashion using photometric technology on the Cobas 8000 modular analyzer series (Roche Diagnostics, UK). The assay is based on a series of enzymatic reactions to initially hydrolyze the triglycerides into glycerol and fatty acids using lipoprotein lipase. For the assay, 2 μL of sample is mixed with 120 μL of triglyceride reagent (TRIGL, Roche) and 28 μL water. The assay takes 10 min with 38 readings taken within this timeframe to assess the increase of the absorbance at 505/700 nm which is proportional to triglyceride content.

*Pause point:* We have used automated analyzer but triglycerides could be quantified manually using other commercial kits on a standard plate reader (i.e., luminescence Triglyceride-Glo™ Assay).

*CRITICAL:* It is important to proceed with this process immediately; it is not recommended to freeze sample before separation of chylomicrons and intralipid. Afterwards, samples could be processed immediately in the same day or could be frozen (−40°C to 80°C) prior to VLDL1 separation.

*CRITICAL:* It is important that the ultracentrifuge tubes are coated with polyvinyl alcohol for optimal performance of density gradient procedures. We used a pre-coated tubes (Seton Scientific, Figure S7), but coating with polyvinyl alcohol can be carried out in-house following standard procedures (Holmquist, 1982).
EXPECTED OUTCOMES

At baseline after the overnight fasted state (10–12 h), VLDL-TG concentration in blood will be steady despite continuous hepatic VLDL-TG export as production will equal tissue uptake. However, during the intralipid infusion, the intralipid particles (Sf > 400) will compete with larger VLDL particles (VLDL1: Sf 60–400) for binding to the lipoprotein lipase enzyme (LPL) (Bjorkegren et al., 1996). As hepatic export of VLDL1-TG continues at a steady rate, a progressive rise in plasma VLDL1-TG level is brought about (Figure S7). Analysis of VLDL1-TG level in each of the sequential blood samples taken during the 75 min infusion permits quantification of the rate of hepatic VLDL1-TG production (Al-Shayji et al., 2007).

QUANTIFICATION AND STATISTICAL ANALYSIS

Calculations

Blood volume in Deciliter (BV) = body weight*0.04*10

Fasting VLDL1-TG= (VLDL1-TG at −15 + VLDL1-TG at +0)/2

VLDL1-TG Production rate (mg/h) = (slope* 87.6)*BV*60

VLDL1-TG Production rate (mg/kg/day) = [(slope* 87.6* BV*60)*24]/body weight

VLDL1-TG pool (mg) = Fasting VLDL1-TG* BV* 87.6

⚠ CRITICAL: For high precision, it is important to calculate the slope and enter it as five decimal places, fasting TG as three decimals, and blood volume (BV) as two decimals. All other numbers with one decimal (Table 4/Figure 3).

LIMITATIONS

MR studies

The disadvantage of using MR is that it is an expensive technique and to initiate the measurements described required specialized staff (MR physicists). However, the MRI scanners are now available in most research centers, and recently MR vendors have introduced options to produce abdominal fat fraction maps at the scanner.
Lipoprotein studies
A limitation of this method is the need to medical expertise for intralipid infusion. Another limitation is the long time to create the density gradient and centrifugation process. However, this could be circumvented by using micro-ultracentrifuge that can save 50% of the required time.

TROUBLESHOOTING
Problem 1
The image quality of MRI is sensitive to respiratory motion. All MR acquisitions require holding breath for 12–16 s. While most people can easily hold breath for this time, some people with breathing difficulties may not. In addition, the pancreas is not well supported within the abdomen, which make it affected by the involuntary movement of other surrounding organs including the stomach and the intestines.

Potential solution
Careful screening by the radiographers of the acquired data. It is important that the participants are trained in breath holding in advance of entering the scanner, and the scan protocol is executed properly. Participants are asked to breathe normally in and out for 3 times before being asked to hold their breath.

Problem 2
Pancreas volume is small and has irregular border in type 2 diabetes (Al-Mrabeh et al., 2016; Al-Mrabeh et al., 2020a). Proper positioning of the MR-opsy in the head, body, and tail of the pancreas requires care when the pancreas is small or with high fat infiltration (Al-Mrabeh et al., 2017).

Potential solution
Although thresholding can overcome the issue of variance between slices as illustrated (Figure 2/Table 3), careful position of the MR-opsy selection is required when the pancreas has high fat infiltration (Figure S8).

The size of the MR-opsy (100 mm²) was chosen to enable easy selection of homogeneous areas at the three main regions of the pancreas (Al-Mrabeh et al., 2017). However, in those extremely small pancreas (<30 cm³), selection could be restricted to one slice only and there could be major overlap between ROIs during the MR-opsy procedures (Figure S9).

Problem 3
For subjects with large BMI, the subject with the phased array coil in position cannot fit into the bore of the scanner.

Potential solution
Where it is permitted, the use of multiple flexible surface coils may be suitable (e.g., on the Philips platform the large and medium flex coils can be combined around the subject’s body and permit high BMI subjects to enter the scanner).

Table 4. Example of raw data on VLDL1-TG production rate in a subject with type 2 diabetes in comparison with a marching non-diabetic control

| Time point (Min) | VLDL1-TG concentration (mmol/L) | Blood volume (dl) | Slope (mmol/L/min) | Fasting VLDL1-TG (mmol/l) | VLDL1-TG Production (mg/kg/day) | VLDL1-TG pool (mg) |
|-----------------|---------------------------------|-------------------|-------------------|--------------------------|---------------------------------|---------------------|
| -15             | 1.3                             | 0                 | 0.014696          | 0.675                    | 741.5                           | 3,117.3             |
| 0               | 1.4                             | +15               | 0.008662          | 0.575                    | 437.0                           | 1,730.7             |
| +20             | 2.2                             | +45               | 52.72             | 3.6                      | 175.5                           | 741.5               |
| +60             | 3.9                             | +75               | 0.675             | 714.5                    | 3,117.3                         | 3,117.3             |
| +90             | 2.9                             | +120              | 3.6               | 714.5                    | 3,117.3                         | 3,117.3             |

Two males aged 59 and 61 years with body weight 131.8 and 85.9 kg, with and without type 2 diabetes, respectively. T2DM: type 2 diabetes; NDC: non-diabetic control.
Problem 4
Very rarely, the fat fractions obtained from the MATLAB script are inverted (pure fat structures are represented as low signal intensity).

Potential solution
This occurs when there is a large displacement of the resonance frequency (a large B0 offset) at the center of the image. The script has a dialog box that allows the issue to be reported and can then force the opposite allocation of water and fat.

Problem 5
Mismatch in FOV or prescription between Dixon and BTFE scans.

Potential solution
Problems in analysis can occur if the two scans have not been exactly been matched for FOV or anatomical location. These should be avoided at the time of installing the protocol. Mismatches in FOV can be dealt with through registration algorithms beyond the scope of this article, but mismatch in prescription will require a rescan of the subject.

Problem 6
Blood withdrawal is not successful.

Potential solution
Finding the blood vessel for overweight people with type 2 diabetes could be sometimes difficult. If this happens, the cannulation procedures should be repeated. The venflon should be flushed frequently (every 5 min), and the arm kept warm with heat packs.

Problem 7
Participant has vaso-vagal episode relating to intravenous cannulae movement.

Potential solution
It is very unusual. The whole procedures of intralipid infusion is medically safe. The participant may feel a little of salty taste due to accumulation of triglycerides in blood. Appropriate screening for trypanophobia is required during recruitment. If participant was uncomfortable, the procedures should be terminated.

Problem 8
The available amount of plasma for lipoprotein separation is less than 2 mL for some time points. The ultracentrifuge tubes need to be balanced accurately during the run.

Potential solution
Make up the volume to 2 mL by addition from 1.006 g/mL density solution. Keep a note of the original plasma volume to work out the correct concentration of VLDL1-TG.

Problem 9
Participant has too much lipid in blood. This is evident to the naked eye as a solid cake of white lipid on the surface after centrifugation of plasma. It would approximate to a plasma total triglyceride concentration far above the normal range.

Potential solution
The protocol suggests collecting 1 mL after ultracentrifugation to remove the VLDL1-TG fraction. However, for some subjects who have high plasma VLDL-TG concentration, it is hard to recover all the lipids in such small volume. The lipids will form a hard to break layer on the top of the tube. In order to solve this, you need (i) break the lipid layer using the fine glass pipette (ii) try to
withdraw some air while taking the lipid layer to create airflow. (iii) wash gently the borders of the tube to remove all the lipids. It is recommended to collect up to 2 mL, and to make a note of the final volume to work out the concentration correctly.

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Roy Taylor (roy.taylor@ncl.ac.uk).

**Materials availability**

This study did not generate any new reagents, all materials are stated in the Key resources table.

**Data and code availability**

This report did not generate new datasets. All datasets generated or analyzed during earlier studies have been reported (Taylor et al., 2018; Al-Mrabeh et al., 2020b). Information about the codes and procedures for MR data analysis will be given upon request from the lead author. Alternatively, there is open source fat-water quantification code available through the International Society for Magnetic Resonance in Medicine (ISMRM): https://www.ismrm.org/workshops/FatWater12/data.htm.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100355.

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**AUTHOR CONTRIBUTIONS**

A.A.-M. wrote the manuscript, optimized the lipoprotein study protocol, and developed the MR-ops method. C.P. carried out the clinical studies and contributed to optimization of the lipoprotein protocol. K.G.H. developed the original protocol for MR data acquisition. R.T. is the principal investigator of the study and edited the manuscript. All authors reviewed and approved the final version of the manuscript.

**DECLARATION OF INTERESTS**

R.T. reports lecture fees from Lilly and Novartis, consultancy fees from Wilmington Healthcare, and is author of the book Life Without Diabetes. A.A.-M. report a grant from Diabetes UK to carry out the Re-TUNE study. All other authors report no competing interests.

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