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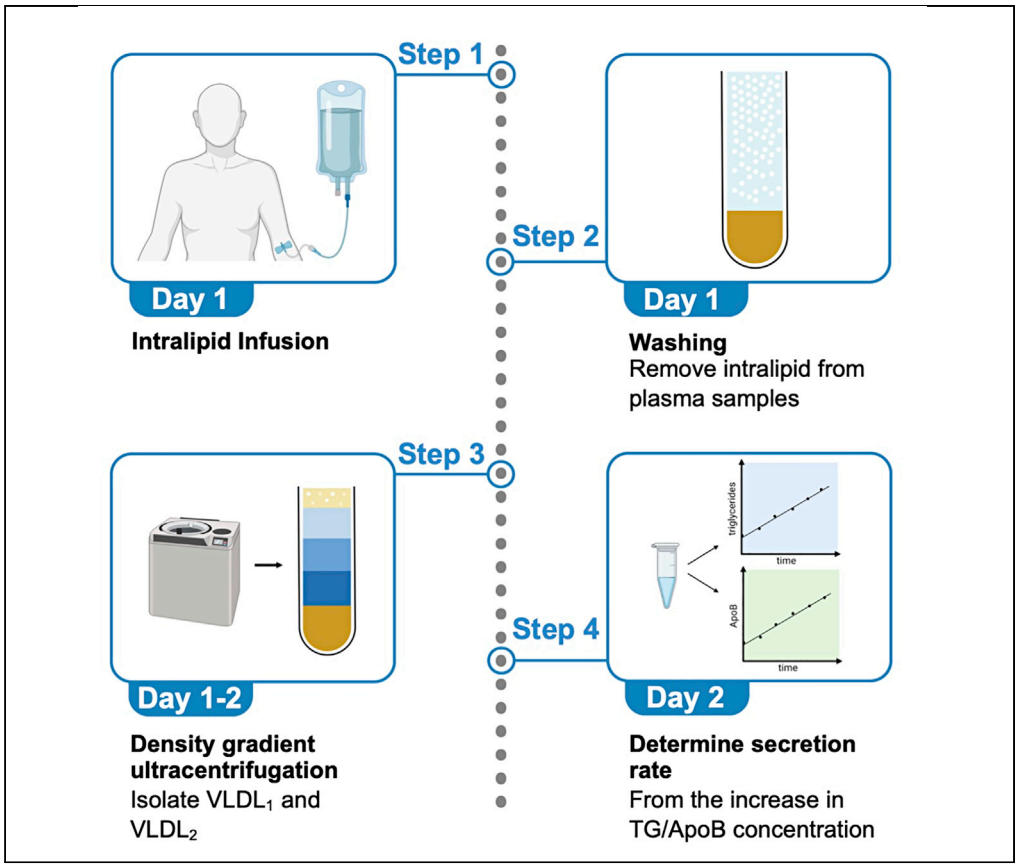
Measuring $VLDL_1$ secretion in humans with an intravenous fat emulsion test



Tracer techniques to assess very-low-density lipoprotein (VLDL) secretion in humans are expensive, are time consuming, and require mathematical models to estimate VLDL kinetics. Here, we describe an alternative, time- and cost-efficient protocol to directly determine $VLDL_1$ secretion with an intravenous (i.v.) lipid emulsion test that does not require tracers and compartmental modeling. We describe steps for intralipid infusion, blood sampling, and removal of intralipid from plasma samples, followed by density gradient ultracentrifugation to isolate $VLDL_1$ fraction and measure the secretion rate.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Matthäus Metz, Clemens Baumgartner, Herbert Stangl, Thomas Scherer

matthaeus.metz@meduniwien.ac.at (M.M.)
thomas.scherer@meduniwien.ac.at (T.S.)

Highlights

Assess VLDL secretion *in vivo* without tracers using an IV lipid emulsion

Isolation of $VLDL_1$ particles with density gradient ultracentrifugation

Time- and cost-efficient technique that does not require compartmental modeling

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Measuring $VLDL_1$ secretion in humans with an intravenous fat emulsion test

Matthäus Metz,[1,3,*] Clemens Baumgartner,[1] Herbert Stangl,[2] and Thomas Scherer[1,4,*]

[1]Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna, 1090 Vienna, Austria

[2]Institute of Medical Chemistry, Center for Pathobiochemistry and Genetics, Medical University of Vienna, 1090 Vienna, Austria

[3]Technical contact

[4]Lead contact

*Correspondence: matthaeus.metz@meduniwien.ac.at (M.M.), thomas.scherer@meduniwien.ac.at (T.S.)
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SUMMARY

Tracer techniques to assess very-low-density lipoprotein (VLDL) secretion in humans are expensive, are time consuming, and require mathematical models to estimate VLDL kinetics. Here, we describe an alternative, time- and cost-efficient protocol to directly determine $VLDL_1$ secretion with an intravenous (i.v.) lipid emulsion test that does not require tracers and compartmental modeling. We describe steps for intralipid infusion, blood sampling, and removal of intralipid from plasma samples, followed by density gradient ultracentrifugation to isolate $VLDL_1$ fraction and measure the secretion rate.

For complete details on the use and execution of this protocol, please refer to Bjorkegren et al. (1996),[1] Al-Shayji et al. (2007),[2] and Metz et al. (2022).[3]

BEFORE YOU BEGIN

Timing: 30 min to 1 h (for step 2)

The protocol below describes step-by-step how to determine hepatic $VLDL_1$ secretion rates using an intralipid infusion test coupled with density gradient ultracentrifugation in fasted humans. The method is based on the observation that intralipid forms chylomicron-like micelles in the blood that compete with triglyceride-rich $VLDL_1$ hydrolysis on the lipoprotein lipase (LPL) with higher affinity and blocks $VLDL_1$ particle breakdown. This leads to the accumulation of large liver-derived $VLDL_1$ (S_f 400-60) particles in the blood. After isolation by density gradient ultracentrifugation, the triglyceride (TG) and apolipoprotein B (ApoB) content of the $VLDL_1$ fraction can be measured and the respective secretion rates calculated from their increase over time. With the use of Intralipid®, a chylomicron-like emulsion, this test follows a similar principle used in animal models where VLDL secretion is often measured after a tyloxapol infusion, a drug which also blocks VLDL utilization by LPL. The presented method therefore allows to directly translate findings from preclinical animal studies to clinical trials. Furthermore, it does not require mathematical modeling.

The protocol can be divided in four main parts: 1) intralipid infusion and blood sampling, 2) removal of intralipid from plasma samples ("washing"), 3) density gradient ultracentrifugation (isolation) and 4) measuring TG and ApoB content in the isolated $VLDL_1$ fraction. We recommend to consider the following steps in advance:

1. Several days prior to the intralipid infusion, make sure that participants receive detailed diet and exercise instructions to increase comparability and reproducibility.

a. Provide national diet recommendations including simple and palatable recipes to the participants.

Note: Ideally, participants should consume an isocaloric diet with accurate macronutrient amounts (e.g., 55% carbohydrates, 25% fats, 20% protein).

b. Ask the participants to follow these recommendations at least 3 days prior to the intralipid infusion test.

Note: Individuals on special diets, such as ketogenic diet, should be excluded.

Note: We encourage participants to rest three days prior to the intralipid infusion test.

c. On the day of the Intralipid infusion test, ask the participants to be fasting for at least 12 h so that gut chylomicron secretion cannot interfere with the measurements.

2. For density gradient ultracentrifugation, prepare NaCl solutions with a density of 1.006 g/mL, 1.02 g/mL and 1.065 g/mL in advance.
   a. The solvent should be deionized water.
   b. The mass concentration required for each solution can be extrapolated from conventional density tables (e.g., in the Handbook of Physics and Chemistry[4]).

Note: We highly recommend to verify the density of each solution by using commercially available densitometers. Note that centrifugation takes place at room temperature (i.e., 20°C). The density should also be determined at room temperature.

c. After preparation the solutions can be stored at room temperature for several months provided that they are tightly sealed.

Note: Approximately 120 mL of the 1.006 g/mL NaCl solution and 18 mL of the other solutions are required for one experiment.

This detailed protocol builds upon the intralipid infusion method previously described by Al-Shayji et al.[2]

Institutional permissions

This is a clinical experiment in human participants. Thus, all steps must be performed in accordance with the relevant institutional and national guidelines and regulations. An ethics committee must approve the experiments and all participants must provide written informed consent before taking part in these studies. The data illustrated here were obtained at the Department of Medicine III of the Medical University of Vienna. The local ethics committee of the Medical University of Vienna and the Austrian Agency for Health and Food Safety approved these experiments and written informed consent was obtained from all participants.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sodium chloride	Sigma-Aldrich	S9888
Deionized water	N/A	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Triglyceride FS 10′	DiaSys Diagnostic Systems	Cat# 1 5710
Human Apolipoprotein B/ApoB Quantikine ELISA Kit	R&D Systems	DAPB00
Experimental models: Organisms/strains		
Human (any age or sex)	N/A	N/A
Other		
Intralipid® 20%	Fresenius Kabi	N/A
SW 40 Ti Swinging-Bucket Rotor Package	Beckman Coulter	331301
13.2 mL, Open-Top Thinwall Ultra-Clear Tube, 14 × 89 mm	Beckman Coulter	344059
Optima L-100 XP ultracentrifuge	Beckman Coulter	N/A

MATERIALS AND EQUIPMENT

In the following protocol, we used the lipid emulsion Intralipid®, a Beckman SW 40 Ti Swinging Bucket Rotor and a Beckman Coulter Optima L-100 XP ultracentrifuge to isolate the VLDL particles.

Alternatives: Other commercially available lipid emulsions (e.g., Liposyn®) likely saturate the lipoprotein lipase as well. However, we like to emphasize that only Intralipid® has been validated with tracer dilution techniques.[1] It is possible to convert this protocol for other swinging bucket ultracentrifugation rotors (e.g., Beckman Coulter SW 41 Ti) using online rotor converters. However, other geometries of the ultracentrifugation tubes (length, width) can affect the separation of lipoproteins. The choice of the ultracentrifuge does not affect the protocol.

STEP-BY-STEP METHOD DETAILS

Part 1: Intralipid infusion and blood sampling

Timing: 1.5–2 h

The intralipid primed-continuous infusion leads to the accumulation of $VLDL_1$ particles over time. Blood samples will be drawn at baseline and every 15 min from an indwelling venous catheter for 75 min (Figure 1).

1. Prepare the intralipid infusion.
   a. Calculate the required amount of intralipid based on the body weight (BW) of the participant. You will require a bolus injection of 0.1 g intralipid 20%/kg BW and a continuous infusion of 0.1 g intralipid 20%/kg BW/h over 75 min.
   b. Aspirate intralipid 20% into an appropriate syringe and assemble it with line and infusion pump.
   c. Place an indwelling venous catheter into both forearms of the participant (one for sampling, one for infusion).
   d. Take a baseline blood sample (without intralipid) from one catheter and block it with an appropriate stopper that prevents blood clotting. Connect the second catheter with the infusion line.
2. Start the intralipid infusion with a bolus injection (0.1 g intralipid 20%/kg BW) over 1 min and then set the infusion pump to an infusion rate of 0.1 g intralipid/kg BW per hour immediately afterward.
3. Take blood samples repeatedly every 15 min for 75 min from the second catheter. Centrifuge tubes to collect plasma (10 min at 5,000 g, 4°C) and place on ice immediately.

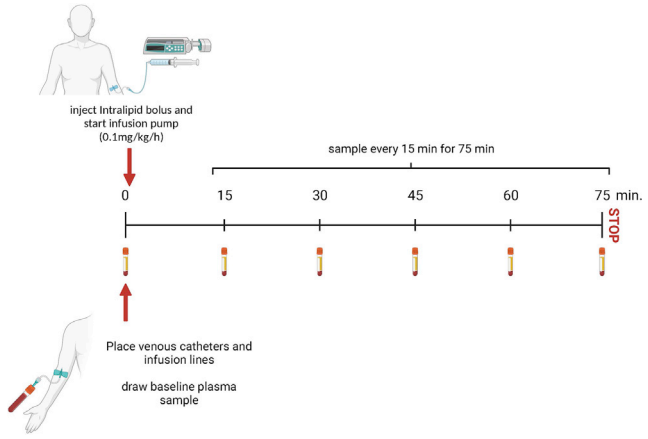

Figure 1. Schematic illustration of the Intralipid infusion test procedure

It starts with baseline blood sampling followed by a primed intralipid bolus (0.1 mg/kg BW) and a continuous infusion (0.1 mg/kg BW/h) to maintain a sufficient lipoprotein lipase saturation. Blood samples are collected every 15 min for 75 min.

Part 2: "Washing" – Removing intralipid

Timing: 3–4 h

This step is designed to remove the intralipid micelles from the freshly collected plasma samples by centrifugation. This major step also incorporates the first step of the density gradient ultracentrifugation.

4. Increase the density of all plasma samples collected at point 3 to 1.1 g/mL.
   a. Pipette 6 mL of plasma into new conical tubes with a cap.
   b. Add 0.678 g solid NaCl to each tube and place on a roller mixer until NaCl is completely dissolved.

   Note: The amount of solid NaCl is based on the finding that plasma has a density of approximately 1.027 g/mL.[5] The amount of NaCl required to raise the density to 1.1 g/mL can be extrapolated / estimated from the conventional density tables (e.g., in the Handbook of Physics and Chemistry[4]).

   Optional: To check the density, it can be measured either with a densitometer or by simply weighing 1 mL of plasma. Small deviations of +/- 0.05 g/mL are unproblematic. If the density is < 1.09 g/mL, it should be corrected.

5. First wash step.
   a. Pipette 5.5 mL of plasma samples with 1.1 g/mL density into an ultracentrifugation tube.
   b. Carefully overlay the plasma with pre-prepared NaCl solution with a density of 1.006 g/mL until the tube is almost completely filled.
   c. Place the tubes into the rotor buckets and balance their weight by addition or removal of 1.006 g/mL NaCl solution. Firmly close the rotor buckets and place the rotor with the buckets into the ultracentrifuge.

   Note: Avoid rapid movements leading to spillover of liquid in the buckets.

   d. Start ultracentrifuge at a speed of 7,600 RPM (equals 7,310 g) for 30 min at 20°C with slow break.

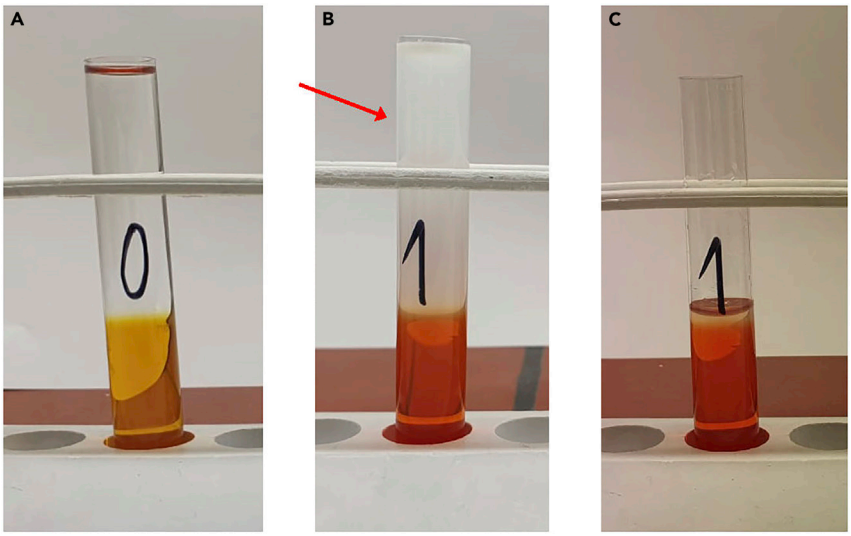

Figure 2. Plasma sample before and after the first washing step

(A) Baseline blood sample without any intralipid.

(B) Blood sample 15 min after the start of the intralipid infusion. The turbid supernatant contains the intralipid micelles.

(C) Intralipid-containing supernatant discarded.

Optional: Centrifugation should be carried out at the temperature at which the density of the NaCl solutions corresponds to the target values mentioned above. It is also possible to perform the centrifugation at 4°C. However, keep in mind that the density depends on the temperature and adjust the density of NaCl solutions accordingly.

Note: This step is designed to float only intralipid micelles. It can be done more quickly at a higher centrifugation force (e.g., 10,000 RPM / 12 656 g for 17 min). Do not use a very high speed as this might result in the isolation of lipoproteins with a sedimentation rate Sf < 400!

e. After the spin, carefully take out the tubes and aspirate the white, intralipid containing supernatant (Figure 2).

6. Second wash step.
   a. Repeat 5b to 5e. After the second wash step, the plasma samples will appear transparent and will only contain negligible amounts of intralipid.
   b. Carefully aspirate 4.5 mL of intralipid-free plasma from the bottom of the tube using a syringe and a long needle, thin plastic tube or a Pasteur pipette tip.

Pause point: Intralipid-free plasma samples can be stored at 4°C overnight (i.e., 10–12 h).

Part 3: Density gradient ultracentrifugation

Timing: 5–5.5 h

This step is designed to separate chylomicrons (hardly detectable in fasted humans), large, triglyceride-rich $VLDL_1$ and small, cholesterol-rich $VLDL_2$ particles. Choose one of the following techniques to build the density gradient: 1) Layering decreasingly dense solutions on top of the previous one or 2) the solution with the lowest density is pipetted into the tubes first and the next densest layer is introduced with a thin syringe or needle at the bottom of the tube.

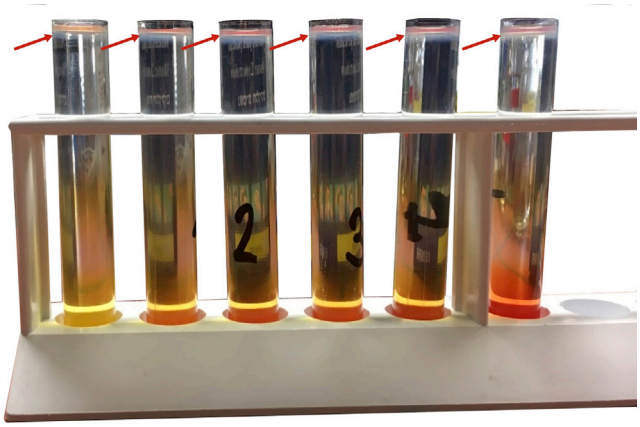

Figure 3. Density gradient ultracentrifugation
The yellowish turbid layer on top contains the $VLDL_1$ fraction.

7. Pipette 4 mL of Intralipid-free plasma with a density of 1.1 g/mL into ultracentrifugation tubes.
8. Carefully overlay the intralipid-free plasma with 3 mL of 1.065 g/mL dense NaCl solution followed by 3 mL of 1.02 g/mL NaCl solution and 3 mL of 1.006 g/mL.

⚠ CRITICAL: The densities of these solutions should be controlled with a densitometer. This is especially relevant for the last layer as it determines the lipoprotein fraction that will be isolated. Lipoproteins that are denser than 1.006 g/mL will not appear on top of the tubes after centrifugation. This layer guarantees that only VLDL particles (and chylomicrons) will be isolated.

9. Carefully assemble the tubes and the rotor as described in step 5c. Start the ultracentrifugation at 40,000 RPM / 202,496 g for 32 min at 20°C.

Note: We recommend using a brake in this step as it reduces working time and results in identical running periods for all experiments but it might cause slight disturbances in the density gradient.

10. Remove the top 1 mL of the gradient containing the residual intralipid fraction.

Optional: To ensure that no $VLDL_1$ particles have been isolated, the triglyceride content in the upper 1 mL can be measured. Since chylomicrons are rapidly cleared from plasma in humans,[6] the amount of triglycerides in this fraction should be minimal after an overnight fast of 12 h.

11. Overlay the gradient with 1 mL of 1.006 g/mL dense NaCl solution and continue the centrifugation with 40,000 RPM / 202,496 g for another 3.5 h.

Pause point: There is a possibility to perform this step overnight. After the set centrifugation time has elapsed, the rotor can decelerate without a brake. This causes the rotor to swing out slowly at low speeds, which prevents the sample from mixing with the NaCl solution.

12. After you remove the tubes from the rotor, you will see the $VLDL_1$ fraction on top of the density gradient (Figure 3). Using a thin pipette tip, aspirate the $VLDL_1$ fraction during constant axial rotation of the tube. We recommend to aspirate 1 or 1.5 mL of the upper layer as it ensures that the $VLDL_1$ fraction is removed completely.

Optional: After collection the $VLDL_1$ fraction, the gradient may further be overlaid with a 1.006 g/mL dense NaCl solution, put back into the rotor and spun for another 14 h at 40,000 RPM / 202,496 g to isolate the cholesterol-richer, smaller $VLDL_2$ fraction. This step can be done for quality control purposes.

Part 4: Determination of the $VLDL_1$-TG and $VLDL_1$-ApoB secretion rate

Timing: 5–6 h

In the final step, measure the amount of $VLDL_1$ triglycerides and/or $VLDL_1$-ApoB and calculate the secretion rate from the linear slope (m) of the increasing concentrations.

13. Measure $VLDL_1$-TG and/or -ApoB concentrations using appropriate commercially available colorimetric assays or ELISAs, respectively (e.g., Triglyceride FS 10′ from Diasys Diagnostic System, Cat# 1 5710, and Human Apolipoprotein B/ApoB Quantikine ELISA Kit from R&D Systems, Cat# DAPB00).

Optional: Normalize the $VLDL_1$-TG content to the plasma volume used.

14. The secretion rate can be calculated by using the following formula first proposed by Al-Shayji et al.[2]:

$$VLDL - TG\ secretion\ rate\left[\frac{mg}{h}\right] = m\left[\frac{mg}{dl \times min}\right] \times plasma\ volume\ (dl) \times 60$$

Note: Plasma volume (l) is approximately 4% of total body mass (kg).[7] As an alternative to calculating the secretion rate, use the slope (m) alone as an equivalent of the $VLDL_1$-TG / ApoB secretion.

EXPECTED OUTCOMES

After $VLDL_1$ isolation (step 12), a clear separation of plasma should already be visible, which becomes even more evident after isolation of the $VLDL_2$ fraction. Both lipoprotein fractions are readily visible after centrifugation, with the $VLDL_1$ fraction usually appearing as an approximately 1–2 mm wide yellowish cloudy layer at the top (Figure 3).

Measurement of $VLDL_1$-TG typically provides a nearly perfect linear increase in triglyceride levels (Figure 4A) and therefore allows a simple calculation of the TG secretion rate. $VLDL_1$-ApoB show stronger fluctuations due to the higher measurement variability of commercial ELISAs compared with colorimetric TG assays yet a linear model can be used here as well (Figure 4B). However, the $VLDL_2$ fraction, since it contains only small amounts of TG, is hardly affected by the intralipid infusion and should therefore remain constant or tend to decrease as an expression of remnant clearance (Figure 4A).

In our previous study, the $VLDL_1$ secretion rates determined by the intralipid infusion test ranged between 49 to 717 mg/h. However, in obese individuals secretion rates up to 2,500 mg/h were reported.[2] These results are well within the range reported with tracer dilution techniques.[2,8,9]

LIMITATIONS

The protocol described here offers a simple, cost- and time-efficient approach to assess $VLDL_1$ secretion *in vivo* in humans. This advantage allows it to be combined with other test/readouts within the same study day in the same participant. However, this protocol works through a competitive

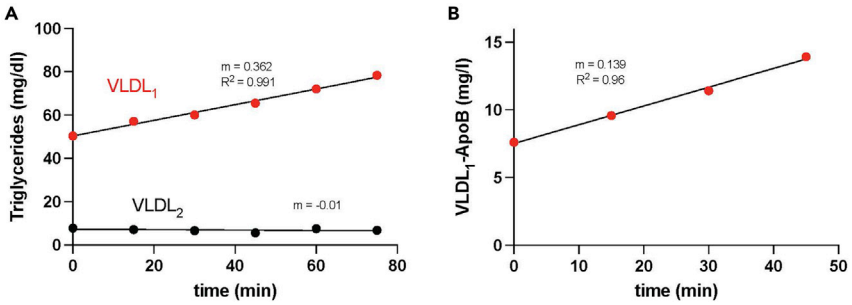

Figure 4. VLDL1-TG and -ApoB concentrations of representative participants

VLDL1-TG and -ApoB concentrations of representative participants. Red dots indicate $VLDL_1$ and black dots indicate $VLDL_2$, m represents the slope of the curves which can be used to calculate secretion rates.

mechanism, i.e., the competition between large intralipid micelles and large $VLDL_1$ particles at the lipoprotein lipase. Pharmacologically, the effect of a "competitive agonist" (i.e., Intralipid in this case) is influenced by the concentration of the "endogenous agonist". High serum triglycerides levels therefore likely influence the accuracy of the method. However, this is probably also dependent on individual factors such as LPL activity, affinity of intralipid micelles, and positive and negative regulators of LPL on VLDL particles (e.g., apolipoprotein CII, CIII, AV).

Finally, VLDL secretion and β-oxidation are substrate dependent processes and are stimulated by an increased lipid supply.[10,11] Thus, the intralipid infusion likely influences these metabolic pathways. However, this limitation occurs always independent of the investigated intervention. However, one should consider this limitation when performing multiple metabolic tests on a study day.

TROUBLESHOOTING

Problem 1

The plasma mixes with the NaCl solution, especially when creating the density gradient (step 8).

Potential solution

Mixing indicates that either the turbulences occurred during pipetting or the plasma density is too low.

Ensure that plasma has an exact density of 1.1 g/mL. This can be confirmed prior to the gradient preparation by weighing 1 mL. Density of intralipid-free plasma should not change after the washing procedure.

Carefully pipette the NaCl solutions. You can take advantage of the adhesion by rotating the tubes slightly during pipetting.

Problem 2

After ultracentrifugation, liquid is missing from one or more tubes (steps 10 and 12).

Potential solution

Make sure that the tubes are properly serviced. Replace the gasket if it is worn or porous. In case the upper layer of the tube is missing, samples should be discarded.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thomas Scherer (thomas.scherer@meduniwien.ac.at).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or code. Representative data derived from Metz et al.[3]

ACKNOWLEDGMENTS

This protocol was prepared jointly with Herbert Stangl (Institute of Medical Chemistry, Center for Pathobiochemistry and Genetics, Medical University of Vienna, Vienna 1090, Austria). We sincerely thank him for his continuous support and knowledge input. The establishment of this protocol was supported by the Austrian Science Fund (FWF) grant no. KLI782 (awarded to T.S.).

AUTHOR CONTRIBUTIONS

Data curation, M.M.; Methodology, M.M., T.S., C.B.; Supervision, T.S.; Writing-original draft, M.M., Writing – review & editing, T.S., C.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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