Metabolite profiles during an oral glucose tolerance test reveal new associations with clamp-measured insulin sensitivity

Christoph Nowak¹, Johan Sundström², Samira Salihovic¹, Andrea Ganna³, Xia Shen⁴, Corey D. Broeckling⁵, Jessica Prenni⁵, Christian Berne⁶, Vilmantas Giedraitis⁷, Johan Ärnlöv²,⁸, Lars Lind², Tove Fall¹*, Erik Ingelsson¹,⁹*

¹Department of Medical Sciences, Molecular Epidemiology and Science for Life Laboratory, Uppsala University, Uppsala, Sweden
²Department of Medical Sciences, Cardiovascular Epidemiology, Uppsala University, Uppsala, Sweden
³Massachusetts General Hospital, Harvard Medical School and Broad Institute, Boston, Massachusetts, USA
⁴Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden
⁵Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, Colorado, USA
⁶Department of Medical Sciences, Clinical Diabetology and Metabolism, Uppsala University, Uppsala, Sweden
⁷Department of Public Health and Caring Sciences, Geriatrics, Uppsala University Uppsala, Sweden
⁸School of Health and Social Studies, Dalarna University, Falun, Sweden
⁹Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, California, USA

*indicates equal contribution

Corresponding author:
Erik Ingelsson, MD, PhD, FAHA
Professor of Molecular Epidemiology
Department of Medical Sciences
Box 1115, 751 41 Uppsala, SWEDEN
E-mail: erik.ingelsson@medsci.uu.se
Phone: +46-70-7569422

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Abstract

Impaired insulin sensitivity (IS) is a major risk factor for cardiovascular disease and type 2 diabetes. Metabolomic profiling during an oral glucose tolerance test (OGTT) can reveal early pathogenic alterations in healthy individuals. Our aim was to identify IS biomarkers and gain new pathophysiologic insights by applying untargeted metabolomics to repeated OGTT plasma samples in association with a hyperinsulinemic-euglycemic clamp assessment. We studied 192 metabolites identified by non-targeted liquid chromatography/mass spectrometry in plasma samples taken at 0, 30, and 120 min during an OGTT in 470 non-diabetic 71-yr-old men. Insulin sensitivity was associated with 35 metabolites at one or more time points in multivariable-adjusted linear regression. The trajectories of nine metabolites during the OGTT were related to IS, six of which (oleic and palmitoleic acid, decanoyl- and dodecanoylcarnitine, deoxycholate-glycine and hexose) showed no associations with IS in the baseline fasting state. The strongest effects were detected for medium-chain acylcarnitines, which increased between 30-120 min in insulin-resistant individuals compared to those with normal IS. In this large community sample, we identified novel associations between clamp-measured IS and metabolite profiles that became apparent only after an oral glucose challenge. Associations of differential medium-chain acylcarnitine and monounsaturated fatty acid trajectories with IS provide new insights into the pathogenesis of insulin resistance.
Introduction

Impaired insulin sensitivity (IS) affects up to one third of the U.S. population and is a major risk factor for cardiovascular disease (1, 2). In a significant number of individuals, accompanying pancreatic β-cell failure leads to type 2 diabetes mellitus and associated adverse consequences including blindness, renal failure, and foot ulcers (3, 4). Measures of IS assess either predominantly hepatic or peripheral (i.e. mainly skeletal muscle and adipose tissue) insulin responsiveness and include steady-state fasting indices (e.g., the homeostasis model assessment of insulin resistance, HOMA-IR (5)) and dynamic-state stimulated models (e.g., the minimal model (6)). The hyperinsulinemic-euglycemic clamp technique is a steady-state stimulated measure considered the gold standard for assessing predominantly peripheral IS (7). However, its resource intensiveness commonly precludes its use in large samples (8).

Metabolomics describes the holistic measurement of small molecular compounds (<1,500 Da) in a biological system. Targeted metabolomics measures only a pre-specified set of metabolites, whilst non-targeted metabolite profiling aims to quantify all known and unknown molecules in an unbiased way. Recent high-throughput methods, including liquid chromatography coupled to mass spectrometry (LC/MS), can identify novel biomarkers, generate new hypotheses, and refine disease models in increasingly larger cohorts (9). Apart from improving risk prediction (10), metabolomics offers unique insights into chronic disease pathology, including metabolite signatures associated with impaired IS and progression to diabetes (11). Metabolic challenges like the oral glucose tolerance test (OGTT) used in the diagnosis of diabetes can uncover early pathophysiologic aberrations in otherwise phenotypically similar persons (12).

Small volunteer trials involving repeated blood sampling during an OGTT found differential metabolite profiles according to IS as assessed by proxy measures like fasting insulin level or the OGTT-derived Matsuda index (13-15). Ho and colleagues (16) compared targeted metabolite levels before and after an OGTT in 377 non-diabetic individuals according to HOMA-estimated IS and found a relative increase of β-hydroxybutyrate, isoleucine, and pyridoxate, as well as a decrease in lactate and orotate levels in subjects with impaired compared to normal IS. Using targeted metabolomics, an i.v. glucose challenge, and the insulin clamp method in 17 risk
allele-carriers for type 2 diabetes and 24 control persons, Then and colleagues (17) demonstrated alterations in phospholipid metabolism related to diabetes risk. Zhao and colleagues (14) used untargeted LC/MS in repeated OGTT blood samples in 16 healthy volunteers and found effects of glucose ingestion on fatty acid and carbohydrate metabolism.

Non-targeted metabolomic profiling in 3-point OGTT blood samples has not previously been applied to a large sample of individuals who also underwent clamp-assessment of IS. We used these techniques in a community sample of 470 non-diabetic men with the aim of identifying post-challenge metabolite profiles that are associated with IS and to characterize these metabolites regarding their role in early diabetes pathology.
Results

**Associations between metabolite levels and insulin sensitivity.** After exclusion of 78 prevalent cases of diabetes, 470 men (mean age 70.64 ± 0.61 yr) were included. The workflow of the study and the main results are summarized in Figure 1. Sample characteristics are shown in Table 1. Thirty-five out of 192 annotated metabolites were associated with IS in age and sample quality-adjusted linear models at the Bonferroni-corrected significance threshold (Table 2): seven glycerophospholipids, six glycerolipids, four glycerophosphoethanolamines, six unsaturated fatty acids (FA), four acylcarnitines (AC), two bile acids, and one each of monosaccharides, peptides, saturated FA, steroids, imidazopyrimidine, and propanolol. The strongest associations were observed for monoacylglycerol (MAG) 18:2 (P-value of the likelihood ratio test comparison to the base model, $P = 3.5 \times 10^{-11}$), MAG 18:1 ($P = 7.7 \times 10^{-10}$), lysophosphatidylethanolamine (LPE) 18:2 ($P = 7.7 \times 10^{-11}$), oleic acid ($P = 2.6 \times 10^{-10}$), and hexose ($P = 3.2 \times 10^{-11}$). Additional adjustment for BMI weakened associations with IS, with the strongest reduction for glycerophospholipids and glycerophosphoethanolamines (Table 2). Among non-unsaturated lipids, the strongest associations persisted for MAG, which showed an inverse relationship with IS at individual time points; an observation that contrasted with exclusively positive associations among glycerophospholipids and -ethanolamines. Higher fasting levels correlated with lower IS for glycerolipids, unsaturated FA, and bile acids, whilst the reverse relationship was observed for most glycerophospholipids and -ethanolamines.

**Association between metabolite trajectories and insulin sensitivity.** Figure 2 illustrates changes in metabolite levels comparing individuals in the highest and the lowest quartile of IS, showing little change between 0-30 min with the exception of bile acids, which increased with higher IS between these time points. All other metabolites tended to decrease with higher IS between 30-120 min. We found nine metabolites whose rate of change between the three assessment points was significantly related to IS (Table 3): the unsaturated FAs oleic and palmitoleic acid, the ACs decanoyl- and dodecanoylcarnitine (C10- and C12-carnitine), the bile acid deoxycholate-glycine, hexose, as well as LPE 18:1, LPE 18:2, and LPE 20:4. Apart from deoxycholate-glycine, all showed declining levels with increasing IS. Figure 3 illustrates four trajectories according to quartiles of IS (plots for all 35 metabolites are included in the
Supplementary appendix). In the baseline fasting state, only the three LPE were associated with IS, whilst associations for the other six metabolites became apparent following the glucose challenge (Table 2). The strongest associations among them were detected for dodecanoylcarnitine ($P = 3.4 \times 10^{-7}$) and decanoyl-L-carnitine ($P = 2.0 \times 10^{-6}$).
Discussion

In a community sample of 470 non-diabetic elderly men, we identified nine glucose-stimulated metabolite profiles that were associated with the gold standard measure of peripheral IS. Among the six metabolites that were not related to IS at baseline, the trajectories of decanoyl-L-carnitine C10 and dodecanoylcarnitine C12 during an OGTT showed the strongest associations with IS.

To our knowledge, this is by far the largest study to date combining metabolomics analysis of multiple OGTT blood samples with a hyperinsulinemic-euglycemic clamp measurement of IS. The relative increase in medium-chain AC levels between 30-120 min after a glucose load in individuals with impaired compared to normal IS has not been previously reported. In our study, we quantified AC of all chain lengths and found several short- and medium-chain AC associated with IS at some time point. Yet, only the medium-chain AC decanoyl-L-carnitine and dodecanoylcarnitine were related to IS in their profile over time.

Fatty acid metabolism involves the esterification of cytosolic long-chain FA to acyl-CoA and the subsequent conversion to AC by carnitine palmitoyl-transferase 1 (CPT1) to allow transport into the mitochondria for β-oxidation (18, 19). Liver and muscle are presumed to contribute to the bulk of AC turnover, yet little is known about the relationship between plasma and tissue levels of AC or their effects on adipocytes (20, 21). Raised fasting levels of medium-chain AC have been associated with impaired IS (22, 23) as well as prevalent type 2 diabetes (24), and can induce cellular inflammation linked to impaired insulin signaling in vitro (25). In a study of 1,019 individuals, higher plasma levels of C10-carnitine and C12-carnitine correlated with impaired fasting glucose, impaired glucose tolerance, and type 2 diabetes (26). In our sample of normoglycemic men who were older than participants in comparative studies, we did not detect an association between AC levels and IS at baseline. A possible explanation may be the age-related decline in skeletal muscle mass – the main contributor to AC metabolism.

Competing theories about the role of AC in impaired IS have recently been reviewed (20, 27) and it has been suggested that reduced FA oxidation and increased suppression of CPT1 may lead to the upstream accumulation of cytotoxic lipids (28,
Alternatively, overactive FA oxidation may produce excess lipid intermediaries (including AC) that interfere with insulin signaling (30). Despite their established adverse effects on insulin signaling, inflammatory activation and cellular stress response in skeletal, cardiac, and hepatic cells, there is a paucity of evidence regarding the effect of raised AC on adipocytes (20), notwithstanding the role of adipose tissue in peripheral IS (31). Adipocyte activity is crucial for postprandial glucose uptake as demonstrated in mice by fat-specific overexpression (32) and knockdown (33) of the GLUT4 transporter. Adipocytes modulate IS by secreting adipokines (34) and by pro-inflammatory effects through macrophages (35) and free FA (36). Our finding of a relative increase in plasma C10-carnitine and C12-carnitine in impaired IS leads us to speculate about a possible short-term insulin-sensitizing effect after a carbohydrate challenge that contrasts with the established adverse long-term consequences. Future in vitro studies to address the functional underpinnings are planned.

The association between a relative increase in total hexose levels in individuals with impaired insulin responsiveness is unsurprising and confirms previous findings on fasting levels and diabetes risk (10). The metabolomics platform we used did not allow for further sub-classification of hexose; apart from glucose, other six-carbon sugars like fructose have been associated with reduced IS (37) and diabetes risk (38).

The glycine conjugate of deoxycholate was the only metabolite whose levels increased in individuals with higher IS during an OGTT. In a previous study of 377 non-diabetic persons, a rise in conjugated and fall in unconjugated bile acid levels during an OGTT was observed, although no relationship with HOMA-estimated IS was found (16). Increased fasting conjugated and unconjugated deoxycholic acid concentrations were previously reported as associated with impaired clamp-measured IS (39) and the unconjugated form correlated with worse HOMA index (40). In our study, we did not observe an association between fasting levels and IS. Insulin and glucose are strong postprandial regulators of bile synthesis (41) and bile acids themselves affect carbohydrate and lipid metabolism (42). Thus, a complicated signaling interplay between post-challenge FA and glucose energy utilization involving bile acids is likely and the relative level increase in insulin sensitive
compared to the fall in insulin resistant persons requires functional follow-up studies for explanation.

The short-term increase during the OGTT of the unsaturated FA oleic and palmitoleic acid in impaired relative to high IS has to our knowledge not been observed before. Several small OGTT studies found declining oleate and palmitoleate levels in both obese and lean individuals (14, 15) and one report from 1981 (43) observed declining trajectories in nine diabetic and twelve non-diabetic persons. Inconsistent results on increased fasting levels of oleic acid indicate correlations with improved IS (44, 45), impaired IS (46), or a lack of association (47). The differential post-glucose increase in reduced IS in our study suggests a potential salvatory effect of adipocyte-secreted monounsaturated FA on peripheral IS that may be mediated by glycolytic stimulation of the synthesis-regulating enzyme stearoyl-CoA desaturase-1. Mechanistic studies are needed to test this assumption.

The strengths of our study include the combination of the gold standard measure of peripheral IS, an OGTT, and non-targeted plasma metabolomics in a uniquely large sample, which, however, precluded replication in an independent cohort. As a compromise, we used the conservative Bonferroni correction for multiple testing and may therefore have missed some true positive associations. Exhaustive examination of over 8,000 spectral features from non-targeted testing identified 192 metabolites, but this approach was limited by the availability of in-house and public database reference compounds, as well as the analysis platform’s proclivity for lipid metabolites. Our cohort of 71-yr-old Northern European men provides for the investigation of pathogenic insights in a homogeneous sample limiting confounding effects of age, sex and ethnicity, but leads to unknown generalizability to women and other age- and ethnic groups.

In conclusion, we discovered differential metabolite trajectories during an oral glucose challenge that were associated with clamp-measured IS and provide novel insights into the effect of medium-chain AC on skeletal myocytes and adipocytes as the main determinants of clamp-measured IS, that will be tested in future in vitro studies.
Methods

Sample characteristics and baseline examinations

In 1970-73, 2,322 of 2,841 invited male residents of Uppsala county, Sweden, born between 1920-24 agreed to participate in the Uppsala Longitudinal Study of Adult Men (ULSAM (48)). The present study is based on the subsequent assessment at age 70 yr (August 1991 to May 1995). All men who completed the OGTT and insulin clamp study and did not meet the exclusion criteria of established diabetes, diabetes medication, fasting plasma glucose ≥7.0 mmol/l, HbA1c ≥6.5% (≥48 mmol/mol), and 2-h plasma glucose (OGTT) ≥11.0 mmol/l, were included. Reasons for exclusion are shown in Figure 1. The 75g glucose OGTT in overnight fasted individuals included blood sampling for immediate storage at -80° C at baseline, 30 min, and 120 min. Plasma glucose was measured by the glucose dehydrogenase method (Gluc-DH, Merck, Darmstadt, Germany) and plasma insulin was analyzed by ELISA (Enzymmun, performed in the ES300 analyzer, Boehringer, Mannheim, Germany). Details are available in the Supplemental Methods and online (http://www2.pubcare.uu.se/ULSAM/invest/70yrs/meth70.htm#06).

Measurement of insulin sensitivity

Within one week of the OGTT, participants underwent a hyperinsulinemic-euglycemic clamp assessment (7) slightly modified with a higher insulin infusion rate of 56 mU x min⁻¹ x m² to completely suppress hepatic glucose production (49). In brief, after a 10 min-bolus dose, semisynthetic human insulin was i.v. infused at 56 mU x min⁻¹ x m² body surface area⁻¹ for 110 min. A plasma glucose concentration of 5.1 mmol/l was maintained with a 20% glucose infusion titrated to plasma values measured at 5 min intervals. The IS index (M/I ratio in mg x kg⁻¹ x kg BW⁻¹ x min⁻¹ per mU/l x 100) quantifies the amount of glucose metabolized per unit of plasma insulin.

Non-targeted metabolomic profiling and feature detection

Details of laboratory procedures and data processing have been described previously (50, 51) and are detailed in the Supplemental Methods. Briefly, blood samples taken
at 0, 30, and 120 min during the OGTT and stored at -80°C until analysis were analyzed by untargeted ultra performance liquid chromatography/mass spectrometry (UPLC/MS) on an Acquity UPLC system coupled with a Xevo G2 Q-TOF MS (Waters Corporation, Milford, MA, USA) featuring an atmospheric electrospray interface operated in positive ion mode. An Acquity UPLC BEH C8 analytical column was injected with non-consecutive duplicates of 1 µl samples. Full scan mode MS analysis (m/z 50–1,200 at 5 scans per s) was performed alternatively at collisions energies of 6 V and 15-30 V. Raw data were processed with XMCS (52) in R and adjusted for retention time correction, analysis date, sample collection, and plate effect as detailed in the Supplemental Methods. Features characterized by m/z ratio and retention time were annotated using in-house reference compounds and public databases according to Metabolomics Standards Initiative guidelines (53). We could identify 192 metabolites in ULSAM.

Statistics

Association testing. In a linear regression framework, we used likelihood ratio tests to find metabolites whose additively combined levels across all three time points explained significantly more variance in clamp M/I than a baseline model adjusting for age and sample quality indicators (storage time, previous thawing, sample type, and possible hemolysis; described in detail in the Supplemental Methods). The conservative Bonferroni-corrected significance threshold was applied to all comparisons to limit the number of false positive findings, as we are not aware of any replication samples available to study the research question at hand. As outlined in Figure 1, significant metabolites were then tested in age- and sample quality-adjusted linear models combining the rates of change between 0-30 min and 30-120 min for trajectory associations with IS. For plots, raw metabolite residual levels adjusted for age and quality covariates were used. Model assumptions of normality and homoscedasticity were ascertained in residual-by-fitted value and normal Q-Q plots. Outlier inspection was done in histograms, and plots of studentized residuals and Cook’s distance. Statistical analyses were performed using the \texttt{lm()} and \texttt{anova()} functions in R.
**Study Approval**

The study was carried out in accordance with the Declaration of Helsinki. The Ethics committee at Uppsala University, Uppsala, Sweden, approved the study and all participants provided written informed consent prior to inclusion.

**Author Contributions**

EI conceived of and EI, TF, and CN designed the study. EI, JS, LL, CB, TF and JÄ secured funding, and EI, JS, LL, JÄ, and VG organized testing in ULSAM. AG, EI, TF, SS, JS, XS, and CN planned, and AG, SS, and CN carried out all analyses. SS, JP, and CDB analyzed plasma samples by LC/MS. CN drafted, and all authors revised the manuscript.

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Figure 1. Study workflow and main results.

Methods and Results

192 annotated metabolites

35 insulin sensitivity-associated metabolites

9 insulin sensitivity-associated trajectories

18 insulin sensitivity-associated in fasted state (0 min)

16 insulin sensitivity-associated after adjustment for adiposity

Linear model 1: M/I on metabolite levels at 0, 30, 120 min
(adj. for age, storage time, sample quality)

Linear model 2: M/I on metabolite level change
(adj. for age, storage time, sample quality)

Metabolomics
Liquid chromatography/mass spectrometry

Study flow

n = 567 who underwent 3-point OGTT, clamp testing, and metabolomic profiling

8 missing data for 30 min
3 missing data for 120 min

n = 556 with complete metabolite data

78 with prevalent diabetes

n = 478 non-diabetic persons

8 with incomplete clamp data

n = 470 individuals included

Design

Oral glucose tolerance test (OGTT)

Hyperinsulinemic-euglycemic clamp within one week of OGTT

glucose load (75g)

0* 30* 120* min

*Blood sampling and storage at –80°C

Time delay ~ 20 years

Metabolomics
Liquid chromatography/mass spectrometry

Methods and Results

n = 470 individuals included

8 missing data for 30 min
3 missing data for 120 min

n = 478 non-diabetic persons

8 with incomplete clamp data

n = 556 with complete metabolite data

78 with prevalent diabetes

n = 567 who underwent 3-point OGTT, clamp testing, and metabolomic profiling

Figure 1. Study workflow and main results.
Metabolite change in the highest quartile of IS

- Increase
- Decrease

Metabolite change in the lowest quartile of IS

- Increase
- Decrease

Figure 2. Change in metabolite levels over 4 min in the most insulin-sensitive individuals. Metabolite levels are adjusted for age and sample quality. Change over 4 min in the highest quartile of insulin sensitivity are plotted parameters. Change over 4 min in the lowest quartile of insulin sensitivity. Metabolite levels are decreased most in insulin-sensitive individuals.

Metabolite change in insulin sensitive persons

- Dodecanoylcarnitine
- Dodecanoyl-L-carnitine
- DOCA-glycine
- LPE18:1-
- LPE18:2-
- Oleic acid
- Palmitoleic acid

Metabolite change in insulin resistant persons

- DOCA-glycine
- LPE18:1-
- LPE18:2-
- Oleic acid
- Palmitoleic acid

Chemical Class

- Unsaturated FA
- Bile Acids
- Lipids
- Acylcarnitines
- Other
Figure 2. Change in metabolite levels over time in individuals from the highest and lowest quartiles of insulin sensitivity. Mean differences between three time points during the oral glucose tolerance test are shown for 35 metabolites associated with insulin sensitivity. Metabolite levels are adjusted for age and sample quality parameters and scaled to SD-unit. Change over time in the lowest (n = 117) and highest (n = 118) quartiles of insulin sensitivity is plotted on the x- and y-axis, respectively. Shapes and colors represent chemical classes. Symbol size is proportional to the $P$-value (-log$_{10}$-transformed) of the regression coefficient representing the association between concentration change and insulin sensitivity (larger size indicates more significant association). Annotated metabolites’ trajectories are significantly related to insulin sensitivity in age and sample quality-adjusted linear regression after correction for multiple testing. For example, the unsaturated fatty acids oleic and palmitoleic acid decrease on average over time in the most insulin resistant persons relative to the most insulin sensitive individuals. DOCA, deoxycholic acid; FA, fatty acid; IS, insulin sensitivity; LPE, lysophosphatidylethanolamine.
Figure 3. Selected metabolites with insulin sensitivity-associated trajectories. Bars represent mean age- and sample quality parameter-adjusted metabolite levels (SD-unit) at three time points during the oral glucose tolerance test grouped according to quartiles of the insulin sensitivity index (M/I ratio; in the order of increasing insulin sensitivity). Error bars indicate 95% CI. Q, quartile.
**Table 1. Sample characteristics of 470 non-diabetic men**

<table>
<thead>
<tr>
<th>Traits</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clamp insulin sensitivity index(^A)</td>
<td>5.3 ± 2.4</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>13.6 ± 6.6</td>
</tr>
<tr>
<td>OGTT glucose (area under the curve)</td>
<td>56.0 ± 30.0</td>
</tr>
<tr>
<td>OGTT insulin (area under the curve)</td>
<td>1225.9 ± 749.5</td>
</tr>
<tr>
<td>Current smoker(^B)</td>
<td>20.1%</td>
</tr>
<tr>
<td>At least moderate physical activity</td>
<td>88.3%</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>26.0 ± 3.3</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>93.2 ± 9.5</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>144.0 ± 18.8</td>
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<tr>
<td>Diastolic BP (mmHg)</td>
<td>82.0 ± 9.5</td>
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<tr>
<td>Antihypertensive medication</td>
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</tr>
<tr>
<td>Serum triglycerides (mmol/l)</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Total serum cholesterol (mmol/l)</td>
<td>5.9 ± 1.0</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Lipid medication(^B)</td>
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</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>3.3 ± 4.7</td>
</tr>
</tbody>
</table>

Mean ± SD are given for continuous, proportions for binary variables. \(^A\)Clamp M/I ratio in mg x kg\(^{-1}\) x kg BW\(^{-1}\) x min\(^{-1}\) per mU/l x 100. \(^B\) Missing responses (3.3% for smoking, 1.7% for lipid medication) are counted as “no”. OGTT, oral glucose tolerance test.
Table 2. Metabolites associated with insulin sensitivity

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Annotation</th>
<th>HMDB reference</th>
<th>Class</th>
<th>Overall linear regression</th>
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<td></td>
<td>LRT P-value compared to the baseline model adjusted for age, sample quality</td>
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<td>Membrane glycerol 18:2</td>
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<td></td>
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<td>Eicosatrienoic acid</td>
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<td>HMDB002301</td>
<td></td>
<td></td>
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<tr>
<td>L-Octanoylarnitine</td>
<td>2</td>
<td>HMDB000791</td>
<td></td>
<td></td>
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<tr>
<td>Docosahexaenoic acid gl ycol conjugate</td>
<td>1</td>
<td>HMDB000611</td>
<td>Bile acid</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic acid glycol conjugate related metabolite</td>
<td>3</td>
<td>[NA]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>1</td>
<td>HMDB000591</td>
<td>Simple fatty acid</td>
<td></td>
</tr>
<tr>
<td>Propionyl acid</td>
<td>1</td>
<td>HMDB000599</td>
<td>Nonenal</td>
<td></td>
</tr>
<tr>
<td>Propionyl acid</td>
<td>1</td>
<td>HMDB000599</td>
<td>Nonenal</td>
<td></td>
</tr>
<tr>
<td>Butyryl acid</td>
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<td>HMDB000599</td>
<td>Nonenal</td>
<td></td>
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<tr>
<td>Uric acid</td>
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<td>HMDB00289</td>
<td></td>
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<td>Myo-Inositol acid</td>
<td>1</td>
<td>HMDB000066</td>
<td>Inositol</td>
<td></td>
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<tr>
<td>Glycero-phosphatidyethanolamine</td>
<td>1</td>
<td>HMDB023555</td>
<td>Phospholipid</td>
<td></td>
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<tr>
<td>Cortisol anione</td>
<td>1</td>
<td>HMDB01547</td>
<td>Steroid</td>
<td></td>
</tr>
</tbody>
</table>

Metabolites that explained more variance in insulin sensitivity than the baseline model (combining age and sample quality parameters) at the Bonferroni-corrected significance threshold (P < 2.6 x 10^-14) are shown. Accuracy according to the Metabolomics Standard Initiative. Linear regression combining metabolite levels at 0, 30, 120 min, age, storage time, previous thawing, sample type, and possible hemolysis as covariates. LRT compare the full to the reduced model excluding metabolites. *P-values for the association of metabolite levels with IS at individual time points adjusted for age and sample quality parameters derived from linear regression models. Underlined values indicate positive associations with insulin sensitivity. HMDB, the Human Metabolome Database; LPE, lysophosphatidylethanolamine; LRT, likelihood ratio test.
Table 3. Insulin sensitivity-associated metabolite profiles following the oral glucose challenge

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>F-statistic P-value</th>
<th>Change 0-30 min</th>
<th>Change 30-120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β ± SEM</td>
<td>P-value</td>
<td>β ± SEM</td>
</tr>
<tr>
<td>LPE 18:1</td>
<td>1.2 x 10⁻⁵</td>
<td>-1.06 ± 0.25</td>
<td>2.5 x 10⁻⁵</td>
</tr>
<tr>
<td>LPE 18:2</td>
<td>7.3 x 10⁻⁸</td>
<td>-1.14 ± 0.28</td>
<td>5.0 x 10⁻⁵</td>
</tr>
<tr>
<td>LPE 20:4</td>
<td>3.0 x 10⁻⁴</td>
<td>-0.55 ± 0.21</td>
<td>8.3 x 10⁻³</td>
</tr>
<tr>
<td>Dodecanoyl carnitine</td>
<td>3.4 x 10⁻⁷</td>
<td>-0.24 ± 0.07</td>
<td>8.9 x 10⁻⁴</td>
</tr>
<tr>
<td>Decanoyl-L-carnitine</td>
<td>2.0 x 10⁻⁶</td>
<td>-0.09 ± 0.13</td>
<td>0.466</td>
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<tr>
<td>Oleic Acid</td>
<td>3.3 x 10⁻⁵</td>
<td>-0.31 ± 0.11</td>
<td>6.9 x 10⁻³</td>
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<tr>
<td>Palmitoleic acid</td>
<td>1.7 x 10⁻⁴</td>
<td>-0.41 ± 0.13</td>
<td>1.7 x 10⁻³</td>
</tr>
<tr>
<td>Deoxycholic acid-glycine</td>
<td>1.2 x 10⁻⁴</td>
<td>0.54 ± 0.13</td>
<td>4.7 x 10⁻⁵</td>
</tr>
<tr>
<td>Hexose</td>
<td>3.8 x 10⁻⁵</td>
<td>-0.61 ± 0.29</td>
<td>0.037</td>
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</tbody>
</table>

Based on linear regression models of clamp M/I = (M₃₀-M₀) + (M₃₀-M₃₀); where M₀, M₃₀, and M₃₀ represent metabolite levels at 0, 30, and 120 min adjusted for age and sample quality parameters. Only metabolites associated at the Bonferroni-corrected significance threshold (P < 1.43 x 10⁻³) are shown. LPE, lysophosphatidylethanolamine.
Metabolite profiles during an oral glucose tolerance test are associated with 
clamp-measured insulin sensitivity

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Supplementary appendix

1. Supplemental methods
1.1. Examinations in ULSAM

Cohort description. The Uppsala Longitudinal Study of Adult Men (ULSAM (48)) was started in 1970 \(n = 2,322\) or 81.7 % of eligible residents enrolled) and involves comprehensive assessments repeated every five to ten yrs. The present study is based on the assessment at age 70 yr \(n = 1,221\) or 72.6 % of 1,681 men still alive and residing in Uppsala county recruited). Only individuals that underwent an oral glucose tolerance test (OGTT), hyperinsulinemic-euglycemic clamp assessment, and had metabolomic profiling for the three time-points were eligible for inclusion in the present study \(n = 567\). We excluded individuals with existing diabetes, defined as fasting plasma glucose \(\geq 7\) mmol/l, glycated hemoglobin HbA1c \(\geq 6.5\) % \((48 \text{ mmol/mol})\), use of anti-diabetic medication according to the Swedish Prescribed Drug Register ATC code A10, and/or diagnosis of diabetes according to the Swedish Hospital Discharge Register.

Baseline examinations. Participants underwent detailed assessment including medical questionnaires, anthropometric measurements, and blood sampling. The OGTT was performed in overnight-fasted individuals who ingested 75 g of glucose in 300 ml of water accompanied by blood sampling immediately before (0 min), 30 min, and 120 min thereafter. Insulin sensitivity was measured on a separate day within one week of the OGTT by the hyperinsulinemic-euglycemic clamp technique according to deFronzo and colleagues (7), modified with a higher insulin infusion rate to suppress hepatic gluconeogenesis. In brief, either the left or right forearm was placed on warmed blankets and used for i.v. cannulation in the antecubital fossa (infusions) and dorsum of the hand (blood sampling). Following baseline sampling 40 min after
cannulation, semisynthetic human insulin was given as a bolus dose over 10 min followed by continuous infusion at a rate of 56 mU x min\(^{-1}\) x m\(^2\) body surface area\(^{-1}\) for 110 min to achieve a steady state of hyperinsulinemia. A plasma glucose concentration of 5.1 mmol/l was maintained by repeated glucose measurements every five min and corresponding adjustments of the infusion rate of a 20 % glucose solution. Glucose levels were analyzed in duplicates using a Glucose Analyzer following immediate centrifugation. Steady-state plasma glucose and plasma insulin concentrations were calculated as the mean of all values obtained between 60-120 min. The IS index (M/I ratio in mg x kg\(^{-1}\) x kg body weight\(^{-1}\) x min\(^{-1}\) per mU / l x 100) represents the amount of glucose metabolized per unit of plasma insulin. It was calculated by dividing the glucose disposal index M (the amount of glucose taken up between 60-120 min in mg x kg\(^{-1}\) x kg body weight\(^{-1}\)) by the mean insulin concentration during the corresponding period. Details of other investigations can be found online (http://www2.pubcare.uu.se/ULSAM/invest/70yrs/meth70.htm#06).

1.2. Metabolomic profiling and feature detection

Metabolomic profiling by LC/MS was performed in venous plasma and serum samples obtained at 0, 30, and 120 min during the OGTT using the equipment and procedures described in the main text and elsewhere (51, 54, 55).

*Laboratory methods and data acquisition.* For protein precipitation, 100 µl of thawed blood samples that had been stored at –80 °C were diluted with 400 µl of methanol, stored overnight at –20 °C, and centrifuged for 30 min at 3,800 g and 4 °C. The supernatant was distributed across three 96-well plates, sealed with heat-seal foil, and stored at –20 °C until analysis. Plates were analyzed in randomly ordered batches of two according to autosampler capacity. Injections were randomized by plate order. All samples were analyzed in duplicates, with the second set of injections following the completion of the first set in all samples. Before each batch of two plates was analyzed, instrument maintenance (cone cleaning, mass calibration, and detector gain calibration) was performed followed by injection of a quality control standard. To ascertain acquisition of high quality data, two conditioning and three quality control injections were performed as 1 µl injections of 20 % methanol solutions containing 2 µg/ml each of terfenadine, caffeine, sulfadimethoxime, and reserpine. Standards were assessed for retention time (±0.05 min), mass accuracy (<3 ppm) and signal intensity.
Protein-precipitated samples were analyzed in 1 µl injections on a Waters Acquity UPLC system. A Waters Acquity UPLC C8 column (1.8 µM, 1 mm by 100 mm) was used for separation, which involved a gradient from solvent A (95 % water, 5 % methanol, 0.1 % formic acid) to solvent B (95 % methanol, 5 % water, 0.1 % formic acid). Injections were made in 100 % A, which was held for 0.1 min, ramped to 40 % B in 0.9 min, to 70 % B over 2 min, and to 100 % B over 8 min. The mobile phase was held at 100 % B for 6 min, reset to starting conditions over 0.1 min, and allowed to equilibrate for 5.9 min. Throughout the run, the flow rate was held constant at 140 µl/min. The separation column and sample were maintained at 50 °C and 10 °C, respectively. A Waters Xevo G2 TOF MS fitted with an electrospray source was infused with the effluent. Data were collected in the positive ion mode with a scanning mass-to-charge ratio (m/z) range of 50–1,200 at a rate of 5 per s. Alternate scans were performed in MS mode (collision energy 6 V) and in idMS/MS mode (15 – 30 V). Before sample testing, calibration was performed by infusing sodium formate solution (mass accuracy 1 ppm). The capillary voltage was held at 2,200 V, the source temperature at 150 °C, and the desolvation temperature at 350 °C at an 800 l/h nitrogen desolvation gas flow rate. The quadrupole was maintained at a collision energy of 6 V and the Waters DataBridge software was used to convert raw data to .cdf files.

**Data analysis.** Metabolic features were detected, aligned, grouped, and imputed using the XCMS software package implemented in R (52). The following functions and parameters were used in deviation from the default setting: Peak detection, xcmsSet( ) - `method = “centWave”, ppm = 25, peakwidth = c(2:15), snthresh = 8, mzCenterFun = “wMean”, integrate = 2, mzdif = 0.05, prefilter = c(1,5);` peak alignment, rector( ) – `method = “obiwarp”, plottype = “deviation”;` peak grouping – `group( ) – bw = 2, minfrac = 0.05, max = 100, mzzid = 0.01;` peak filling, fillPeaks-chrom( ). Parameter selection was based on a random selection of 20-40 samples with duplicate or triplicate injections used to evaluate a variety of parameter settings within a reasonable interval around the values suggested by the software developers. We selected parameters that maximized intra-replicate correlations for the final analysis. Chosen configurations were additionally assessed by inspecting the plots generated by the peak detection and grouping algorithms. We detected 10,162 features in ULSAM. Feature intensities were log-transformed and normalized by fitting ordinary least
squares linear regression models for associations between feature intensity and
covariates of unwanted variability (retention time correction, analysis date, sample
collection, and plate effects). The resultant residuals were carried forward for
subsequent analyses. Samples with abnormal total feature intensities and/or low
Spearman correlation coefficients between duplicates were manually scanned for and
removed.

Metabolite annotation. Each metabolite is commonly represented by a cluster of
metabolic features that share a similar retention time but differ in mass according to
the inherent fragmentation pattern of the parent compound. Highly correlated features
with similar retention times were combined to reconstruct representative
fragmentation spectra, which were used for annotation. Metabolites were identified
according to the four-level hierarchical approach suggested by the Metabolomics
Standards Initiative (53), where Level 1 indicates the highest confidence (based on
matching by mass, fragmentation pattern, and retention time to an in-house standard
compound) and Level 4 designates unknown metabolites, which could not be matched
to public compound libraries according to spectral and/or mass and/or retention time
similarities with named metabolites (Level 2) or chemical classes (Level 3).

Exhaustive one-by-one feature evaluation yielded 192 annotated metabolites in
ULSAM.

1.3. Adjustment for sample quality

Adjustment for sample quality parameters. The time lag between sample collection in
the early 1990ies and metabolomic profiling of deep-frozen blood samples in the
early 2010s when the new technology became available required adjustment for
various sample quality indicators based on continuously updated logbooks maintained
by laboratory staff and cohort administrators. As samples were obtained from
consecutive participants over several years, assessment time was controlled for to
avoid confounding with systematic drifts in laboratory practice. It was included as a
continuous variable specifying the time in days between the first and last assessment
date (mean 380±205 d, maximum 749 d, n = 470). We coded blood sample
characteristics in three indicator variables (previous thawing, sample type, and
possible hemolysis) based on laboratory logbook information. In 26 % of samples
(122/470), logbook information implied previous thawing and subsequent re-freezing
(unknown number of times). All specimens, except for two serum samples (0.4 %), were ethylenediaminetetraacetic acid (EDTA) plasma samples. In 2.7 % of cases (8/470) logbook entries pointed to possible hemolysis.
Supplemental Figures. Metabolite profiles according to insulin sensitivity. Bars represent mean age- and sample quality parameter-adjusted metabolite levels (SD-unit) at three time points during the oral glucose tolerance test grouped according to quartiles of the insulin sensitivity index (M/I ratio; in the order of increasing insulin sensitivity). All 35 insulin sensitivity-associated metabolites are presented. Error bars indicate 95% CI. Q, quartile.
Glycerophosphoethanolamines - GPE

Unsaturated Fatty Acids – UFA
Bile Acids - BA

Acylcarnitines – AC
References


