





Best-practice study designs in metabolic research

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### Today's Speakers



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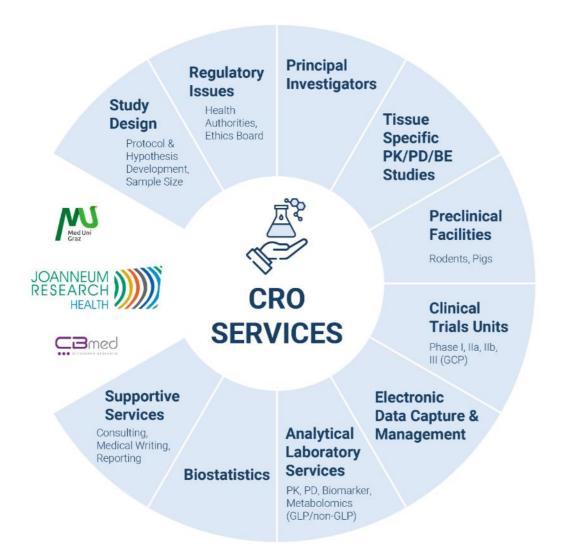


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#### Who We Are

#### Boutique CRO for research projects & drug development programs



- We develop, optimize and validate analytical methods for preclinical/clinical studies and pharmaceutical products.
- We combine scientific expertise with service orientation and high quality standards (GLP/GCP).
- We offer analytical solutions for pharmaceutical product development.

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### Key Learnings



How can analytical methods best support metabolic research?



How to quantify peptides that regulate metabolic processes?



How to use metabolomics in diabetes and obesity research?



How to use stable isotope tracers in clinical studies?



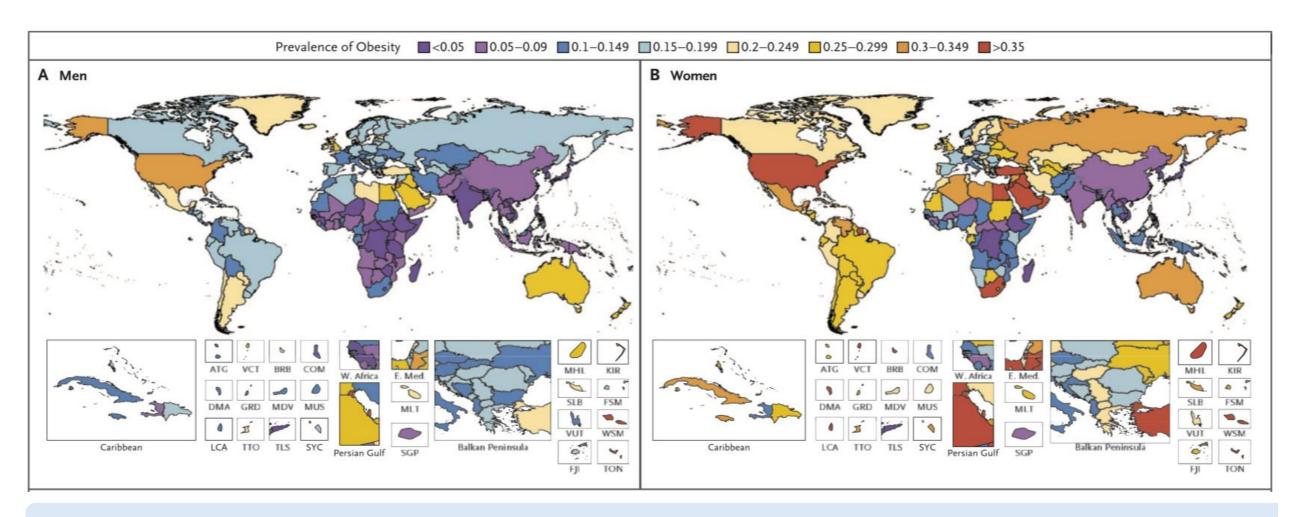


# Obesity is a disease - the Epidemiology





### The Global Tsunami



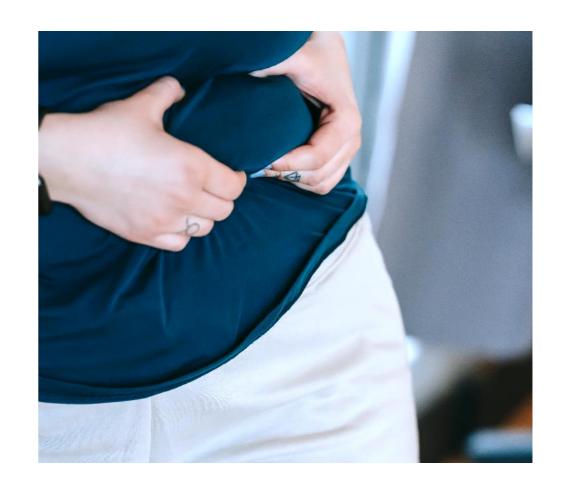






### Obesity is a disease - the Epidemiology

- 2017: 650 million adults with obesity
   (BMI>30) worldwide, 110 million children with obesity worldwide<sup>1,2</sup>
- Higher increase in children than adults<sup>1</sup>
- 1990-2015: Doubling of incidence in most countries<sup>1,2</sup>
- Severe comorbidities that are life-threatening and costly for society



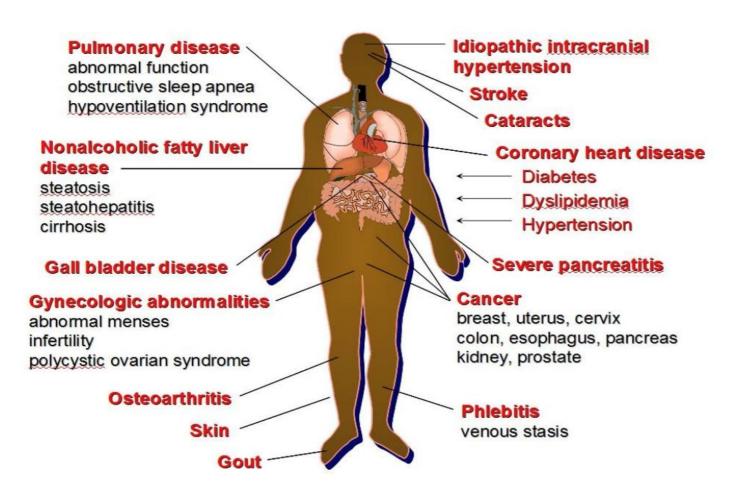
<sup>&</sup>lt;sup>1</sup> The GBD 2015 Obesity Collaborators. *N Engl J Med* 2017;377:13. doi:10.1056/nejmoa1614362

<sup>&</sup>lt;sup>2</sup> World Obesity Federation





# Obesity is a disease



- Mechanical complications
- Metabolic and cardiovascular complications
- Psychological complications
- Cancer



# Increased risk of developing severe and life threatening comorbidities that are costly for society

CVD

Increased risk of cardiovascular disease

Heart failure

Increased risk of heart failure

Sleep apnoea

Increased severity of sleep apnoea

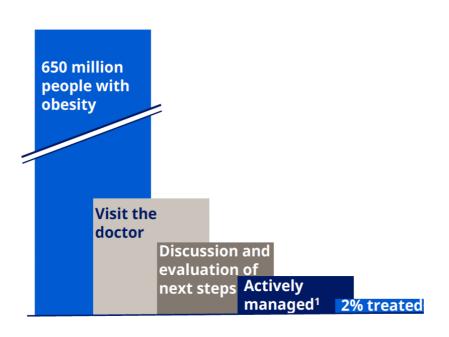
Osteoarthritis

Increased risk of osteoarthritis

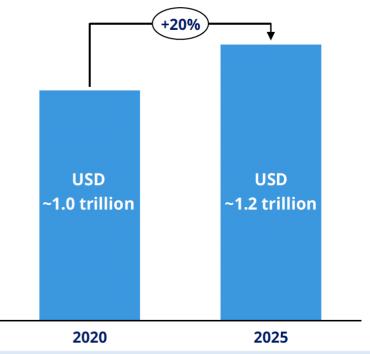
Diabetes

Increased risk of type 2 diabetes

Only 2% of the 650 million people with obesity are treated with medication



Global healthcare costs related to obesity expected to increase by 20% by 2025



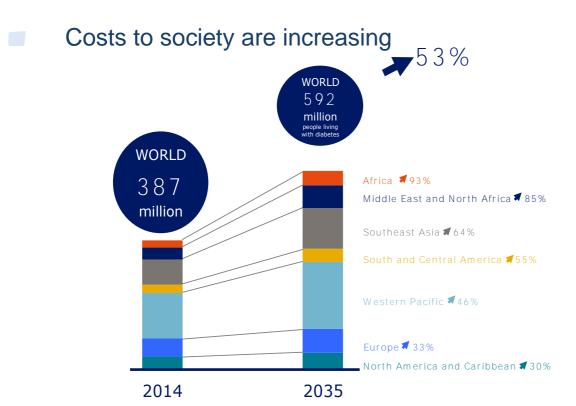




### Obesity

#### The obesity tsunami leads to a fast growing problem in diabetes care

By 2035, diabetes will rise to 592 million







Driven by 200% increased risk of all cause mortality<sup>1</sup>



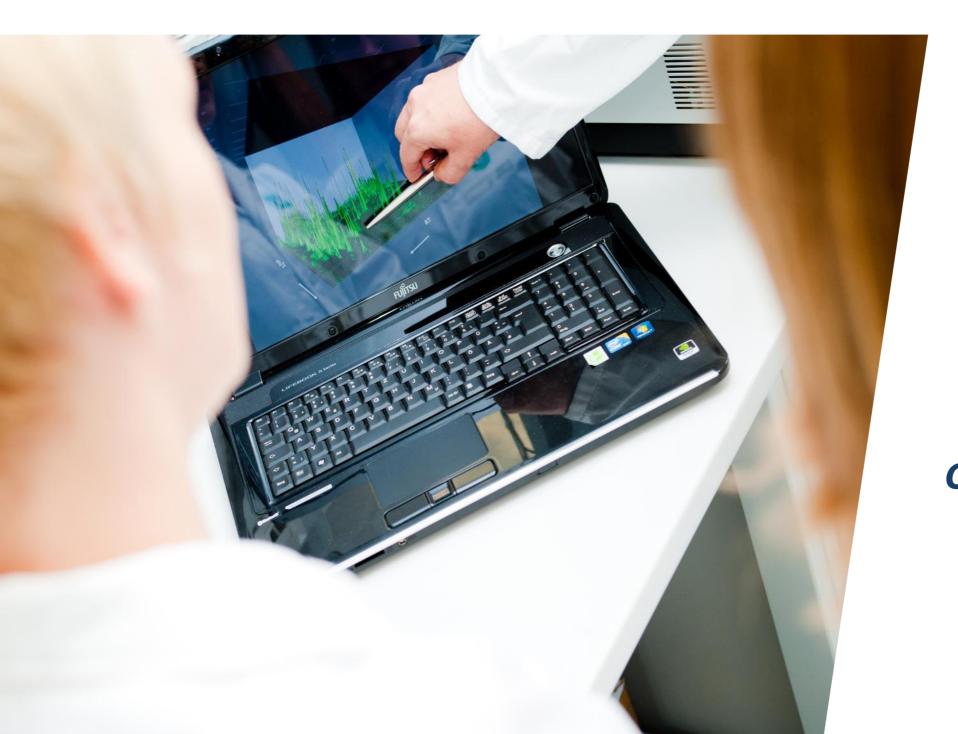
- 70% of people with diabetes die from atherosclerotic CVD<sup>2</sup>
- 150% increase in risk of stroke<sup>3</sup>



Higher likelihood of neuropathy, retinopathy, limb amputation, cancer and cognitive dysfunction<sup>4</sup>



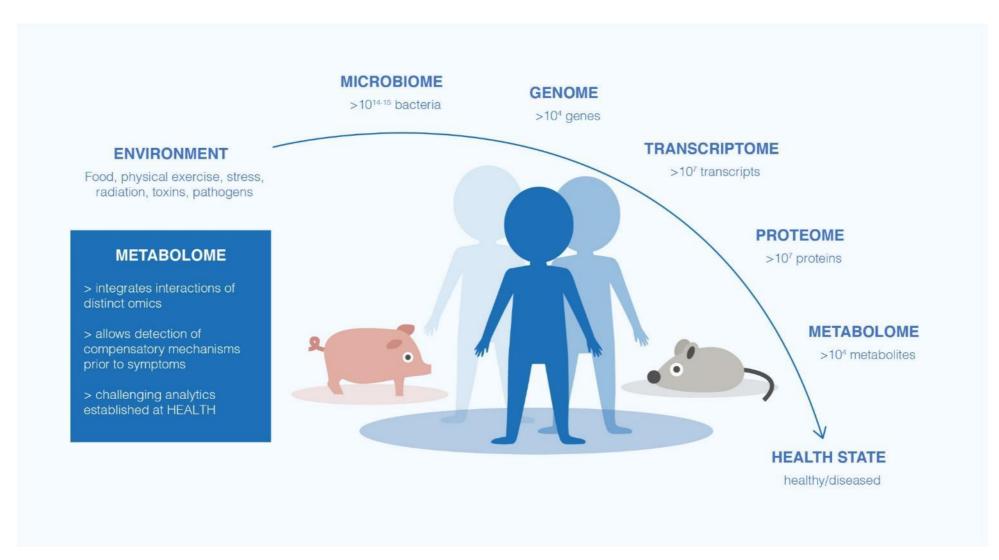




How to use metabolomics in diabetes and obesity research?



### **Metabolomics**





### Metabolomics Workflow

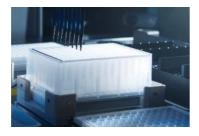
### Sample Generation

- Tissue, tissue biopsies
- Biological liquids
  (blood, urine, interstitial fluid)
- Cell culture & supernatant



### Sample Preparation

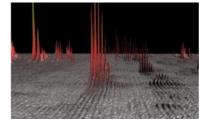
- Extraction of low molecular weight compounds
- Preparation of QC sample



#### **Analysis**

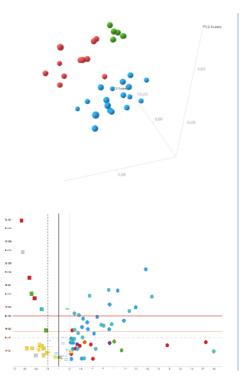
Untargeted High Resolution Mass Spectrometry

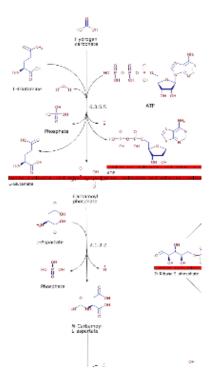




### Data Processing & Statistics







**Biological** 

Credit: https://projects.ig.harvard.edu/files/pfister/files/vis2005\_0.pdf



### Metabolomics Quality Report

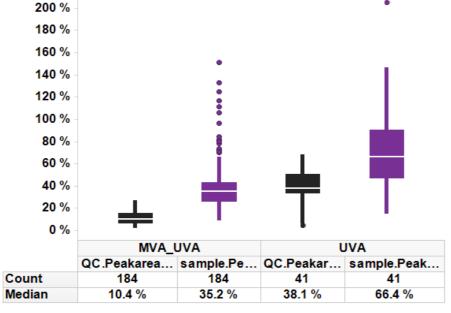
Detected known metabolites: >200

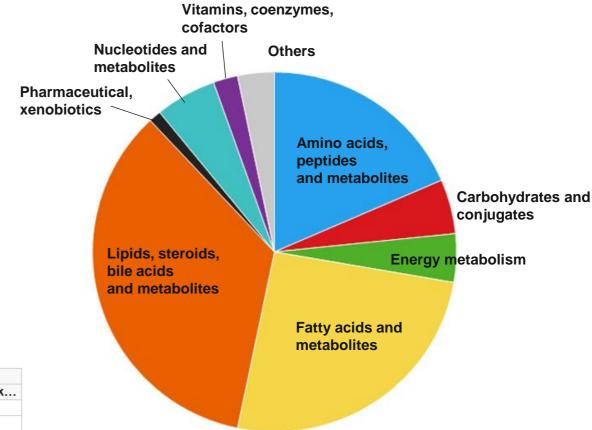
Detected metabolic features: 1500-2000

Quality controls: Median Multivariate statistics

RSD 8-15%

Mass deviation<5 ppm</li>







### © Case Study: Alternate Day Fasting

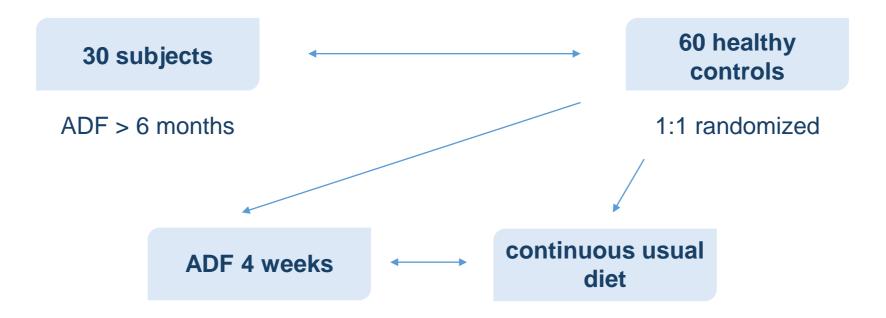
- Can Alternate Day Fasting (ADF) be an alternative to caloric restriction?
- How is metabolism affected by ADF?







### Study Design



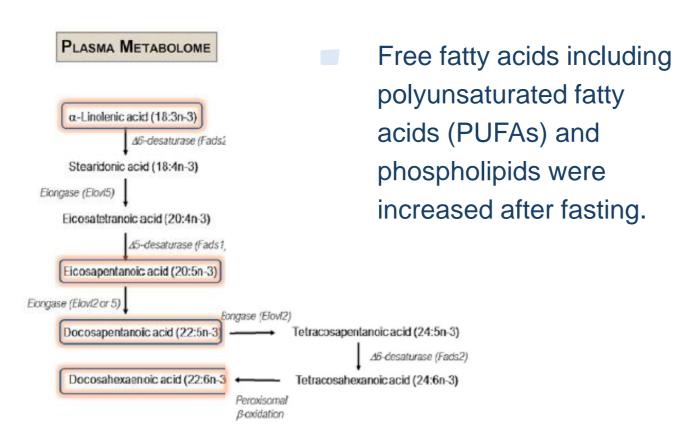
ADF: every 2<sup>nd</sup> day – ad libitum

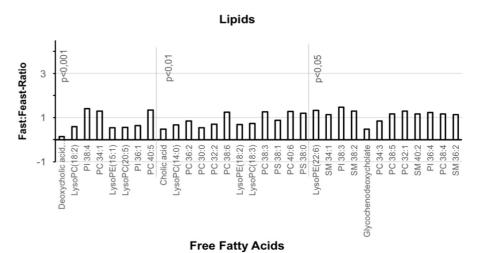
Fasting days: no solid or liquid food, no caloric beverages

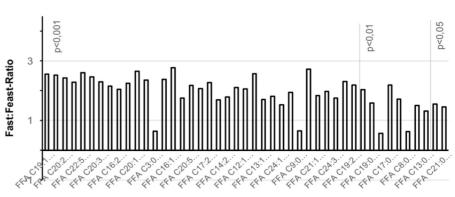




#### Changes between 36 h fasting and 12 h feeding period: Lipids





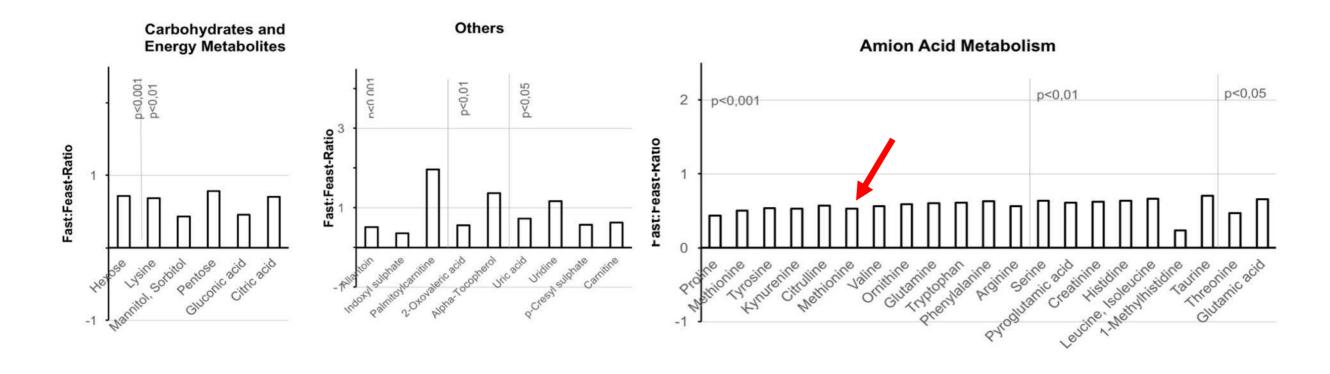






## Changes between 36 h fasting and 12 h feeding period: amino acids, carbohydrates, energy metabolites

Amino acid related metabolites and carbohydrates were at least 20% lower after fasting:







#### Overall results

Our Metabolomics Platform found 113 metabolites significantly changed (54 up and 49 down)

- Periodic shifts towards increased PUFA levels
- b-hydroxybutyrate increased during non-fasted conditions.

Framingham risk score for CVD was significantly reduced after 4 weeks.

My personal challenge:

Lowered blood · pressure by 20 mm Hg



### **Other Case Studies**

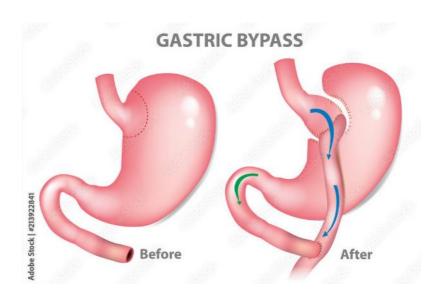
#### RESEARCH ARTICLE

#### An Untargeted Metabolomics Approach to Characterize Short-Term and Long-Term Metabolic Changes after Bariatric Surgery

Sophie H. Narath<sup>1</sup>, Selma I. Mautner<sup>1,2,5</sup>, Eva Svehlikova<sup>2</sup>, Bernd Schultes<sup>4</sup>, Thomas R. Pieber<sup>1,2,5</sup>, Frank M. Sinner<sup>1,2</sup>, Edgar Gander<sup>1</sup>, Gunnar Libiseller<sup>1</sup>, Michael G. Schimek<sup>3</sup>, Harald Sourij<sup>2,5</sup>\*, Christoph Magnes<sup>1</sup>

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Published: September 1, 2016 https://doi.org/10.1371/journal.pone.0161425



#### **Results overview**

- CDV risk associated metabolites decreased after bariatric surgery (BS): Alanine, valine, choline, leucine/isoleucine, phenylalanine, tyrosine
- TMAO elevated after BS





#### Adipocyte Glucocorticoid Receptor Deficiency Attenuates Aging- and HFD-Induced Obesity and Impairs the Feeding-Fasting Transition

Kristina M. Mueller<sup>1,2</sup>, Kerstin Hartmann<sup>3</sup>, Doris Kaltenecker<sup>2</sup>, Sabine Vettorazzi<sup>3</sup>, Mandy Bauer<sup>3</sup>, Lea Mauser<sup>3</sup>, Sabine Amann<sup>4</sup>, Sigrid Jall<sup>5,6</sup>, Katrin Fischer<sup>5,6</sup>, Harald Esterbauer<sup>4</sup>, Timo D. Müller<sup>5,6</sup>, Matthias H. Tschöp<sup>5,6</sup>, Christoph Magnes<sup>7</sup>, Johannes Haybaeck<sup>8</sup>, Thomas Scherer<sup>9</sup>, Natalie Bordag<sup>10</sup>, Jan P. Tuckermann<sup>3</sup> nand Richard Moriggl<sup>1,2,11</sup> n

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Corresponding authors: Richard Moriggl, richard.moriggl@lbicr.lbg.ac.at, and Jan P. Tuckermann, jan.tuckermann@uni-ulm.de.

K.M.M. and K.H. contributed equally to this work, and J.P.T. and R.M. contributed equally to this work.

Diabetes 2017 Feb; 66(2): 272-286. https://doi.org/10.2337/db16-0381



#### **Results overview**

- 59 significantly decreased metabolites in Adipocyte Glucocorticoid Receptor Deficient Mice (GR<sup>△Ad</sup>)
- Decreased metabolites related to fatty acid/lipid metabolism, amino acid metabolites (proteogenic, BCAA)
- Adipocyte GR central role in regulation of homeostasis, feeding/fasting transition
- Promotes obesity and metabolic disorders in fat-fed and aged mice



# Other Selected Metabolomics Studies @ JOANNEUM RESEARCH

Metabolic phenotyping of first degree relatives of patients with type 1 diabetes.	Zügner E, presented at EASD 2017 link to presentation
N-acetylaspartate catabolism determines cytosolic acetyl-coa levels and histone acetylation in brown adipocytes.	Prokesch A, et al. Sci Rep. 2016, 6:23723. doi:10.1038/srep23723
Accumulation of basic amino acids at mitochondria dictates the cytotoxicity of aberrant ubiquitin.	Braun RJ, et al Cell Rep. 2015 ,10(9):1557-1571. doi:10.1016/j.celrep.2015.02.009
Targeting the H3K4 demethylase KDM5B reprograms the metabolome and phenotype of melanoma cells.	Vogel FCE, et al. J Invest Dermatol. 2019, 139(12):2506-2516.e10. doi: 10.1016/j.jid.2019.06.124
Cognitive impairment by antibiotic-induced gut dysbiosis: analysis of gut microbiota-brain communication.	Fröhlich E, Brain Behav Immun. 2016, 56:140-55. doi:10.1016/j.bbi.2016.02.020
Identification of novel metabolic interactions controlling carbon flux from xylose to ethanol in natural and recombinant yeasts.	Trausinger G, et al Biotechnol Biofuels. 2015, 25;8:157. doi:10.1186/s13068-015-0340-x
Lysosomal acid lipase regulates fatty acid channeling in brown adipose tissue to maintain thermogenesis.	Duta-Mare M, et al Biochim Biophys Acta Mol Cell Biol Lipids. 2018, 1863(4):467-478. doi:10.1016/j.bbalip.2018.01.011
Differential effects of SGLT2 inhibitors on mitochondrial oxidative phosphorylation, glucose uptake, cell energy level and metabolism in HEPG2 cells and HUVECS.	Zügner E, presented at EASD 2020 link to presentation
Cardioprotection and lifespan extension by the natural polyamine spermidine.	Eisenberg T, et al Nat Med. 2016, 22(12):1428-1438. doi:10.1038/nm.4222





How to use stable isotope tracers in clinical studies?

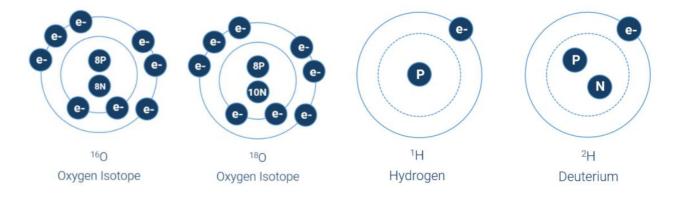


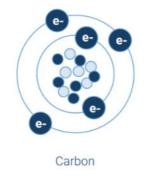


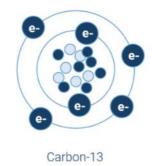
#### Stable Isotope Tracers in Metabolic Research

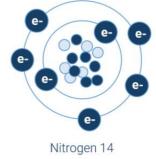
#### **Important Applications**

- Metabolite turn-over rates (in-vivo)
- Lipolysis
- Glucose kinetics
- Body water content and total energy expenditure
- Metabolic pathway tracing





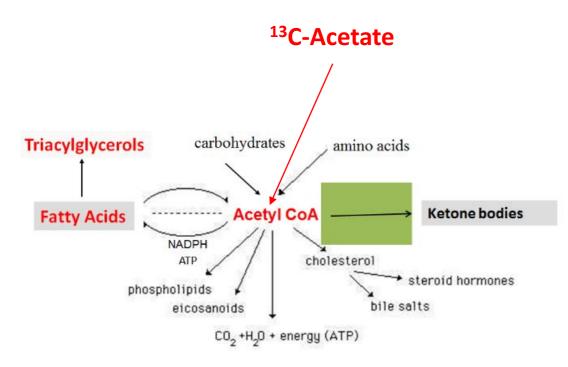


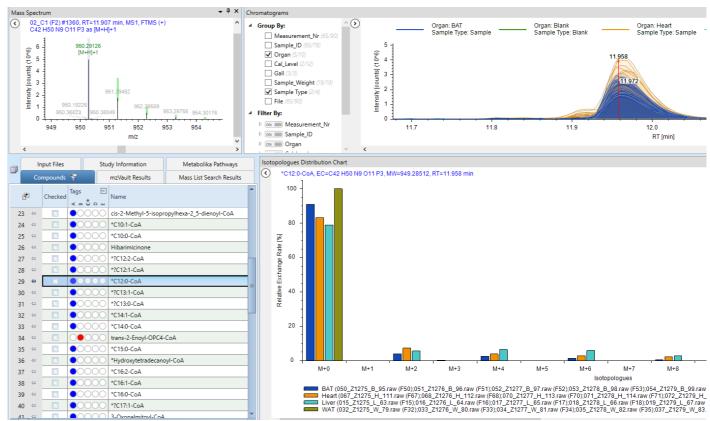






### Metabolomics Supported by Stable Isotope Labelling



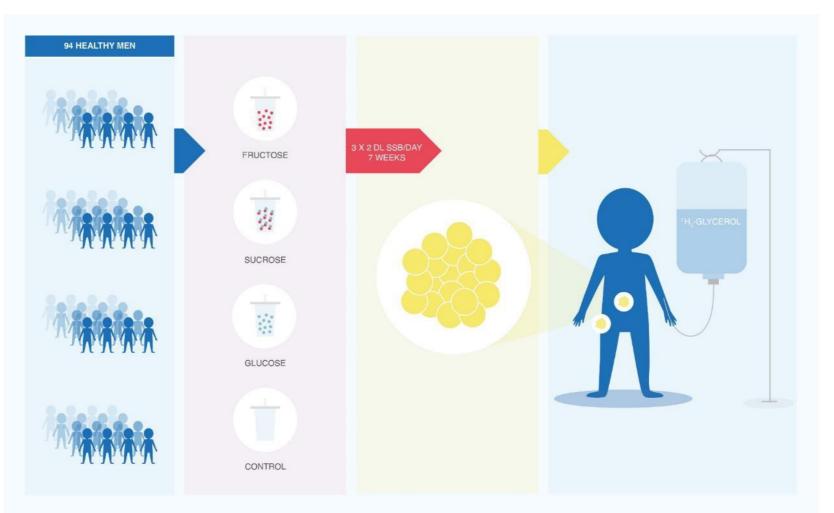






# Case study: Lipolysis determination by glycerol stable isotope tracer



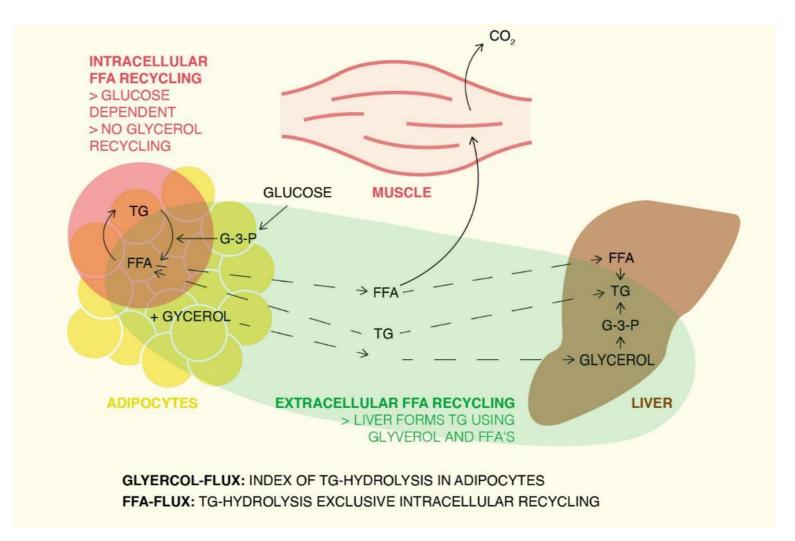








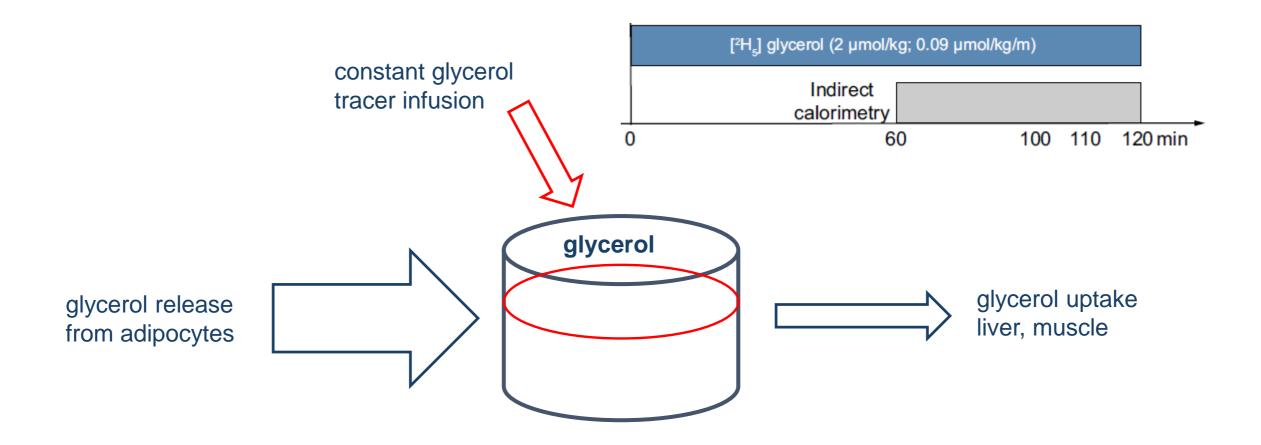
### Peripheral Lipolysis - FFA recycling



Credit: JOANNEUM RESEARCH

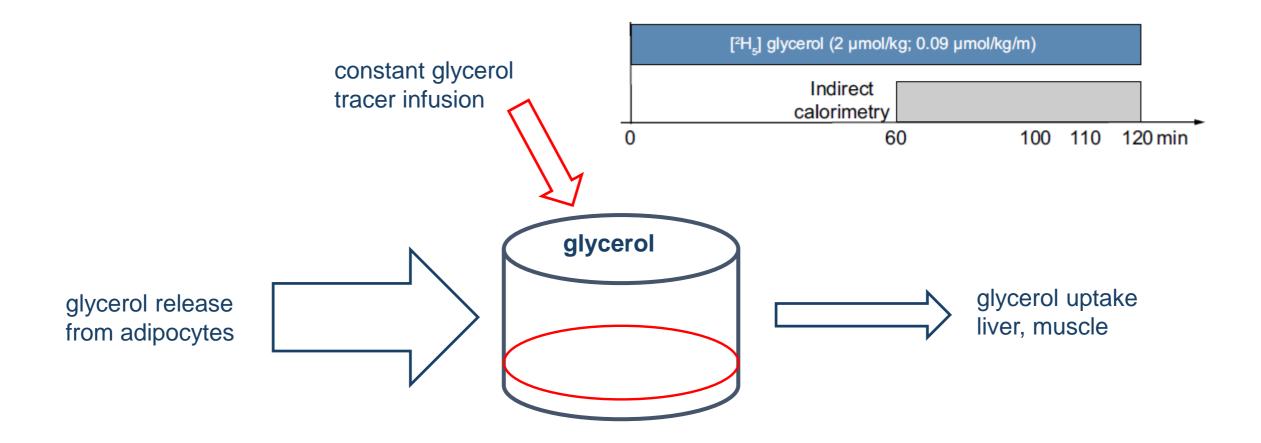


### Glycerol Tracer





### Glycerol Tracer







#### Lipolysis determination by glycerol stable isotope tracer

### Calculations using "One-Pool-Model" (acc. Steel)

$$Ra = \left\{ F - Vd \left[ \left( \frac{C}{1 + \frac{(E_1 + E_2)}{2}} \right) x \left( \frac{E_2 - E_1}{t_2 - t_1} \right) \right] \right\} / (E_1 + E_2) / 2$$

Ra..... rate of appearance of glycerol: measure of peripheral lipolysis

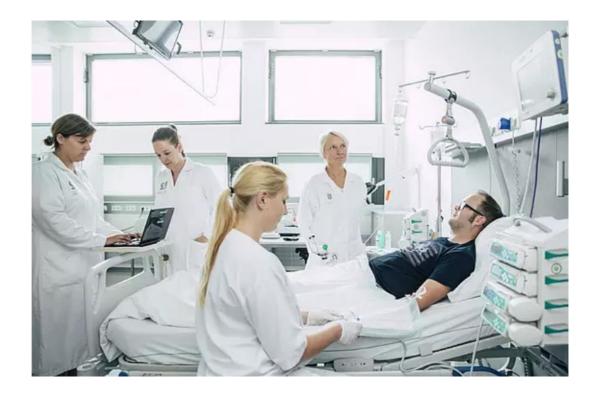
F.....isotope infusion rate: *constant lb (0.1 µmol)* 

Vd.....0.23 l/kg for glycerol

C...... plasma concentration of the tracee: concentration of glycerol

(E2 - E1).....change in enrichment (TTR) between two consecutive samples

(t2-t1).... time between two consecutive samples



Credit: https://zmf.medunigraz.at/oes/oe-fuer-forschungsinfrastruktur/core facilities/einrichtung-zur-durchfuehrung-klinischer-studien

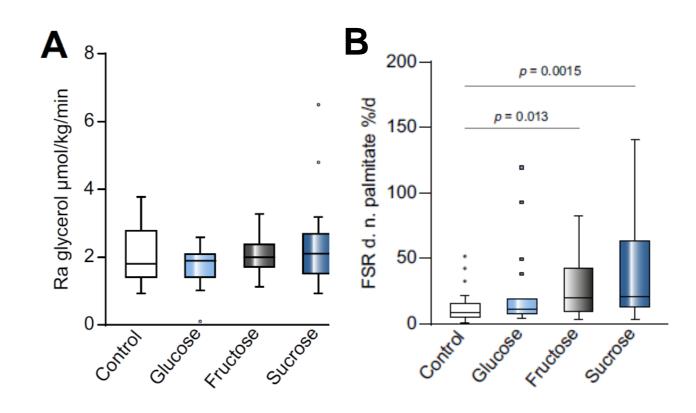




#### Lipolysis determination by glycerol stable isotope tracer

#### **Overview Results**

- No difference in energy intake between groups
- No differences in peripheral lipolysis
- No change in total fatty acids or fatty acid oxidation rate
- 2 fold higher basal hepatic fatty acid synthesis for fructose and sucrose
- Glucose does not increase basal hepatic fatty acid synthesis.





# Other Selected Tracer Studies @ JOANNEUM RESEARCH

Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM.	Zenz, S. J Clin Endocrinol Metab. 2018 Apr 1;103(4):1408-1417. doi: <u>10.1210/jc.2017-01836</u>
Effect of dapagliflozin, saxagliptin, and the combination of both on glucagon, endogenous glucose production (EGP) and glycerol in patients with type 2 diabetes	Sach-Friedl, S. Presented at the EASD 2017 link to presentation
Insulin glulisine, insulin lispro and regular human insulin show comparable end-organ metabolic effects: an exploratory study.	Horvath, K. Diabetes Obes Metab. 2008 Jun;10(6):484-91. doi: <u>10.1111/j.1463-</u> <u>1326.2007.00734.x</u>
A double-blind, randomized, dose-response study investigating the pharmacodynamic and pharmacokinetic properties of the long-acting insulin analog detemir.	Plank, J. Diabetes Care. 2005 May;28(5):1107- 12. doi: <u>10.2337/diacare.28.5.1107</u>
Proportional dose-response relationship and lower within-patient variability of insulin detemir and NPH insulin in subjects with type 1 diabetes mellitus.	Wutte, A. Exp Clin Endocrinol Diabetes. 2007 Jul;115(7):461-7. doi:10.1055/s-2007-976512



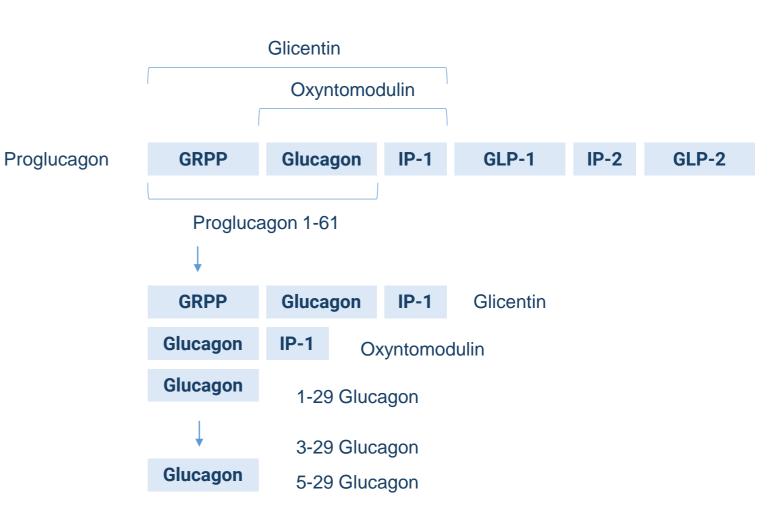


How to quantify peptides that regulate metabolic processes?



### **Quantification of glucagon**

- Insufficient selectivity of assays due to precursors
- Cross-reactivity because of structural similarities with e.g. glicentin, oxyntomodulin
- Active peptide difficult to distinguish from degradation product







### Quantification of glucagon

# Different glucagon concentrations obtained with different assays

Glucagon levels [pmol/L]	Healthy volunteers (n=20)	T1D Patients (n=20)	T2D Patients (n=20)
ELISA	7.0 (5.9 – 8.6)	8.7 (4.3 – 10.9)	11.0 (8.3 – 16.7) *
RIA	66.8 (55.9 – 87.9)	65.9 (49.9 – 84.7)	79.2 (62.5 – 126.1)

<sup>\*</sup>p<0.014 for comparison against healthy volunteers.



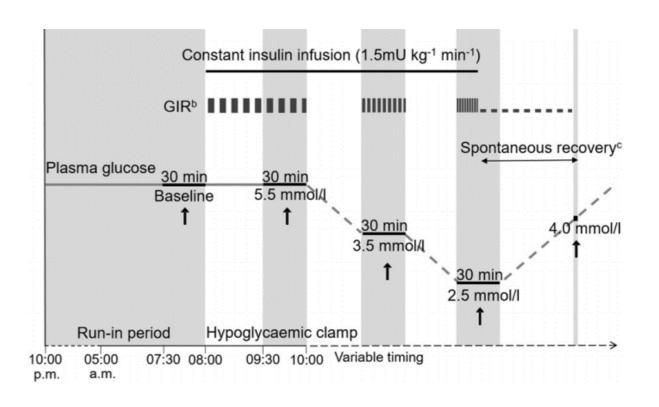


#### Case Study: Glucagon in hypoglycemia



### Response to hypoglycemia in newly diagnosed & long term type 1 diabetes patients

- 10 C-peptide positive and 11 C-peptide negative patients
- Stepwise hypoglycemic clamp at 5.5, 3.5, 2.5 mmol/L
- Glucagon quantification with selective ELISA
- Endogenous glucose production (EGP) determination





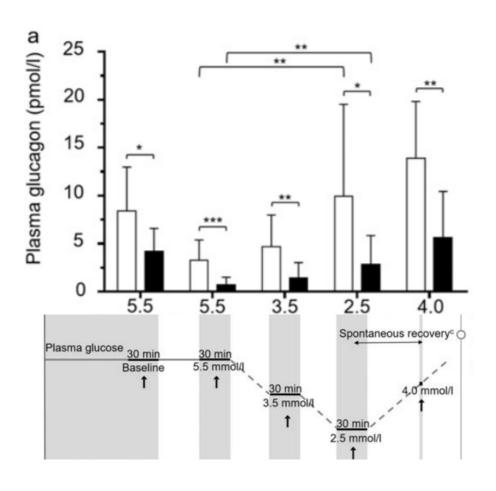




#### Glucagon in Hypoglycemia



### Effect of hypoglycemia on glucagon secretion



- Glucagon levels responded to hypoglycemia in both groups.
- Higher glucagon in C-peptide positive patients
- Glucagon levels in both groups were lower than previously reported.



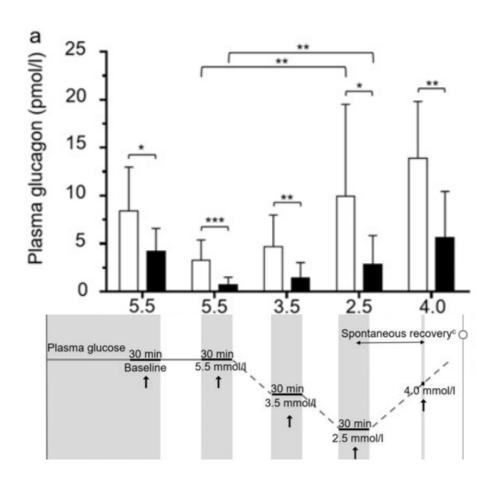




#### Glucagon in Hypoglycemia



### Effect of hypoglycemia on glucagon secretion



- Glucagon levels responded to hypoglycemia in both groups
- Higher glucagon in C-peptide positive patients
- Glucagon levels in both groups were lower than previously reported
- More selective ELISA supported the detection of physiological differences.





# Quantification of insulin and insulin analogues

 Simultaneous quantification of human insulin and insulin analogues for clinical studies





### Quantification of insulin and insulin analogues

### Combing selective and non-selective ELISA

- A non-selective ELISA is used to determine the sum of insulin and insulin analogues.
- A selective ELISA is used to determine human insulin only.
- The concentration of the insulin analogue is calculated.
- Results are validated according to EMA guideline, GLP compliant.

	<b>Human Insulin</b>	Insulin Aspart
LLOQ	3.2 mU/L	6.4 mU/I
ULOQ	201.7 mU/L	101.9 mU/I
Linearity	passed	passed
Accuracy	%Diff= -4%-11%	%Diff= -14%5%
Precision	%RSD= 4%-10%	%RSD= 4%-12%
Specificity	< LLOQ analogue	Cross reactivity 93%-113%
Hook effect	Not observed	Not observed
Parallelism	%RSD= 6%	%RSD=10%
Dilution accuracy	%Diff= 10%	%Diff= 11%















#### Case Study: pharmacokinetics (PK) of insulin formulations



- Patients at constant blood glucose level with human insulin overnight
- At t=0 infusion with investigated insulin formulation
- PK profiles of investigated insulin formulation have to be baseline corrected.







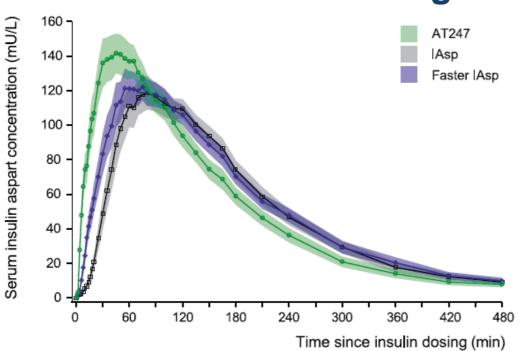
#### PK of insulin formulations

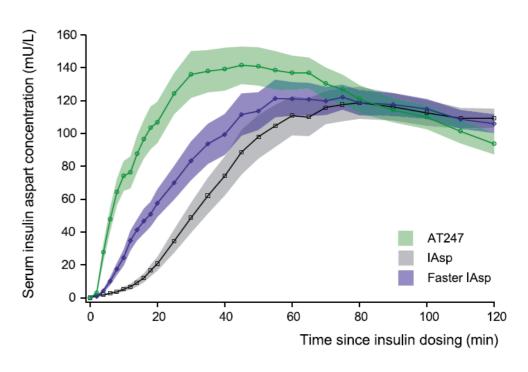


#### A newly developed formulation showed faster PK



#### background corrected PK



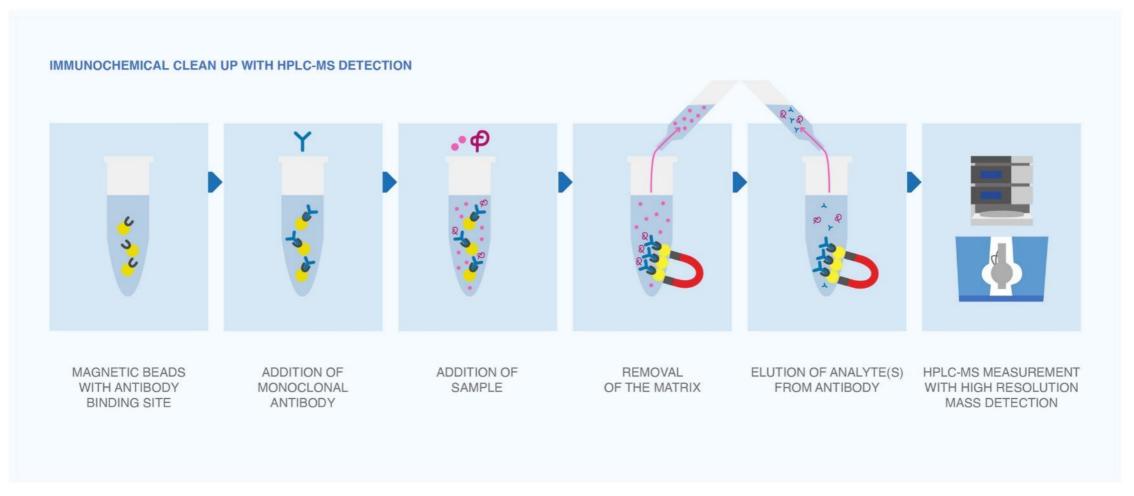






#### Determination of insulin and insulin analogues

### Mass spectrometry for insulin analysis

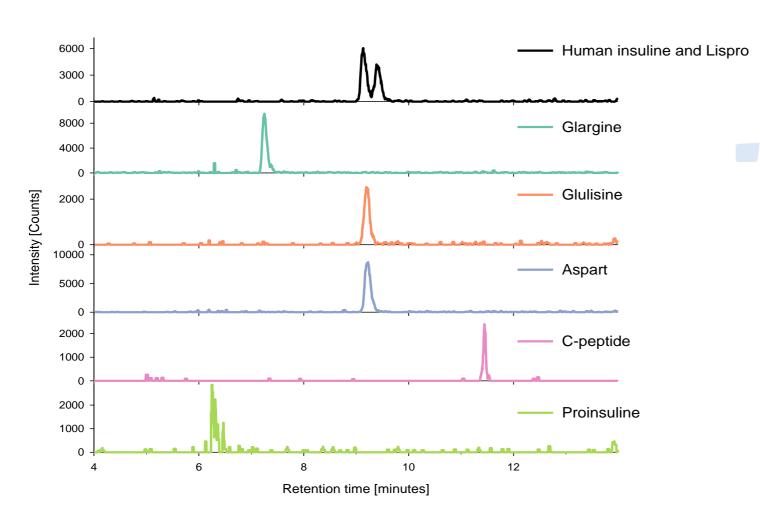


Credit: JOANNEUM RESEARCH



### Determination of insulin and insulin analogues

### Sensitive mass spectrometry for insulin analysis



80 pM standard spiked to insulin free serum

### Key Learnings



- Mass spectrometry-based metabolomics
- Stable isotope tracer methods
- Peptide hormone analysis are advanced analytical methods that support metabolic research.



Mass spectrometry-based metabolomics give a holistic picture of biological changes after interventions.



Stable isotope tracer methods can determine metabolite turnover rates in clinical trials, e.g. for the assessment of lipolysis and glucose kinetics.



High selectivity and sensitivity are key factors to quantify peptides hormones.



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