JOANNEUM RESEARCH HEALTH

Webinar

Best-practice study designs in metabolic research

June 1st, 2021
2 pm London / 3 pm CET

Today's Speakers

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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.

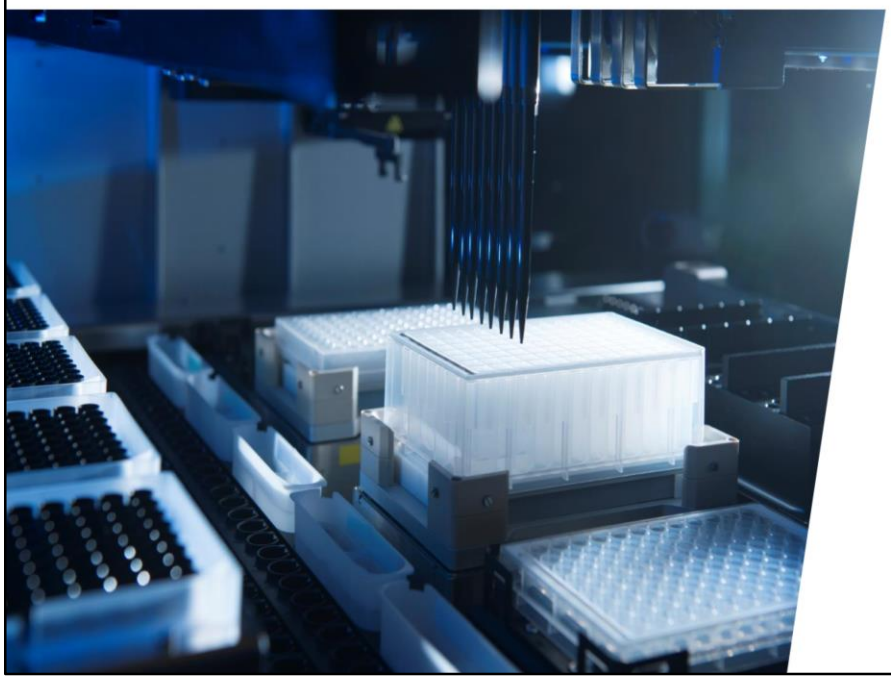
Key Learnings

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?

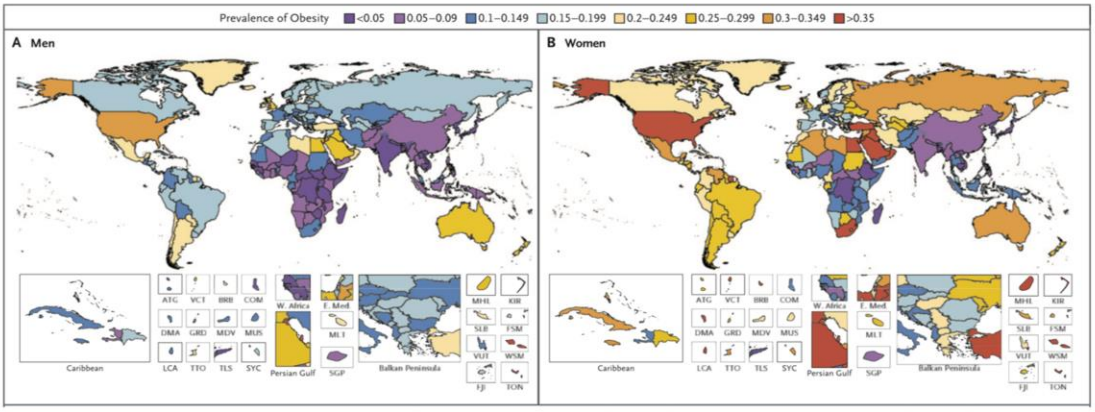
How to use stable isotope tracers in clinical studies?



Obesity is a disease - the Epidemiology

Obesity

The Global Tsunami



The GBD 2015 Obesity Collaborators. N Engl J Med 2017;377:13. doi:10.1056/nejmoa1614362

This is a very brief overview about the role of obesity as a disease and its epidemiology. Unfortunately, obesity is a global tsunami.

This world map shows the prevalence of obesity for men on the left side and for women on the right side. Yellow and red countries have an obesity incidence above 20% showing that obesity is truly a worldwide problem.

Obesity

Obesity is a disease - the Epidemiology

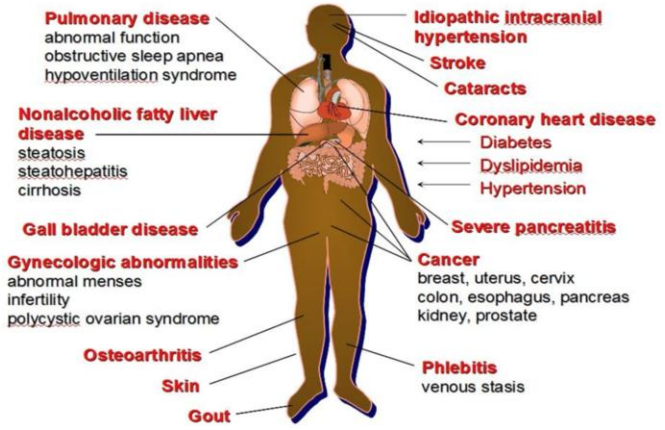
- 2017: 650 million adults with obesity (BMI>30) worldwide, 110 million children with obesity worldwide[1,2]
- Higher increase in children than adults[1]
- 1990-2015: Doubling of incidence in most countries[1,2]
- Severe comorbidities that are life-threatening and costly for society

[1] The GBD 2015 Obesity Collaborators. *N Engl J Med* 2017;377:13. doi:10.1056/nejmoa1614362
[2] World Obesity Federation

In 2017, there were 650 million adults with obesity, which correlates to a BMI, body mass index, of above 30. But there are also 110 million children with obesity worldwide and this is especially concerning because there is a 95% chance of living a life as obese adults for obese children. The higher increase in obesity in children compare with adults, makes this even a more concerning picture. So overall from 1990 to 2015, there was a doubling of the incidence in most countries that collect these data. More importantly, severe comorbidities have been linked to obesity that are life threatening and actually quite costly to society.

Obesity

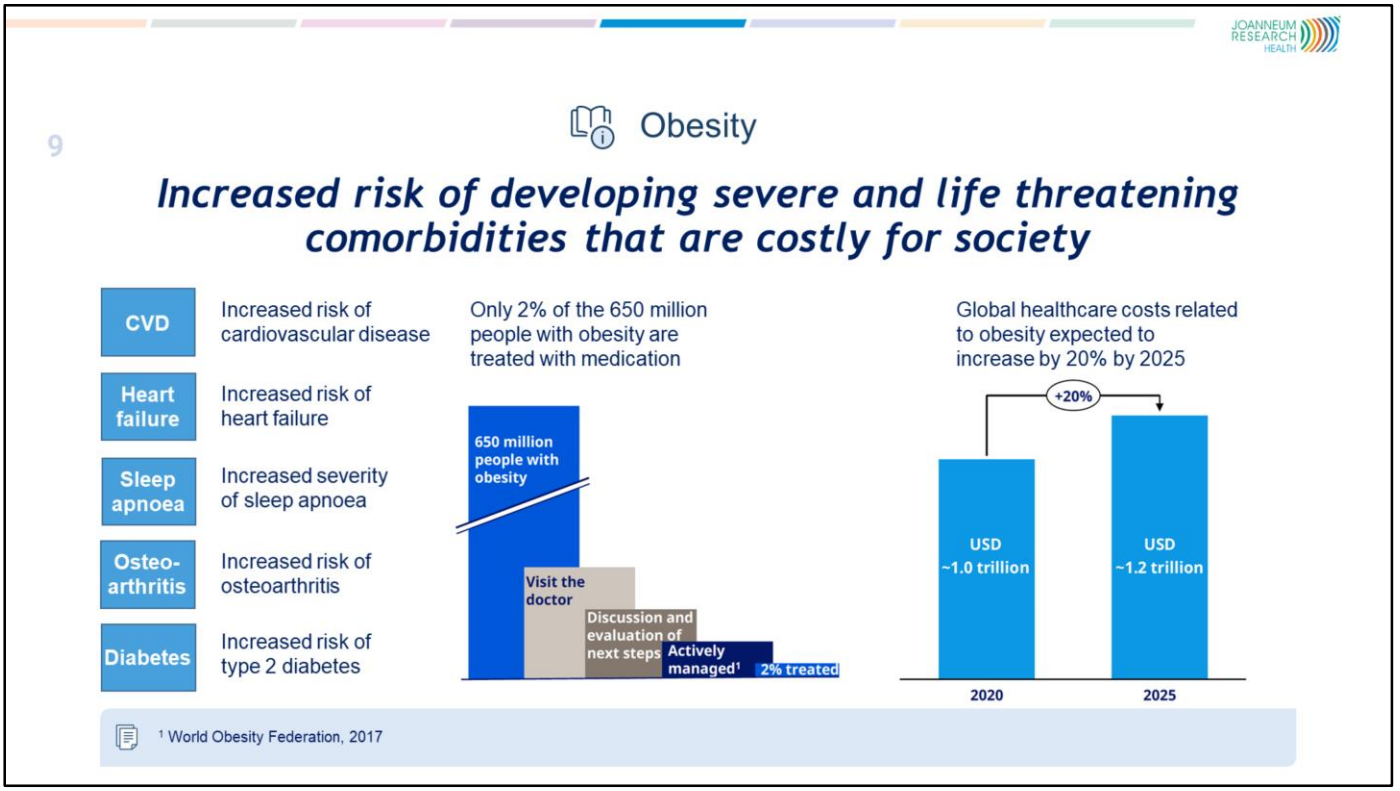
Obesity is a disease



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

The GBD 2015 Obesity Collaborators. N Engl J Med 2017;377:103. doi:10.1056/nejmoa1614362

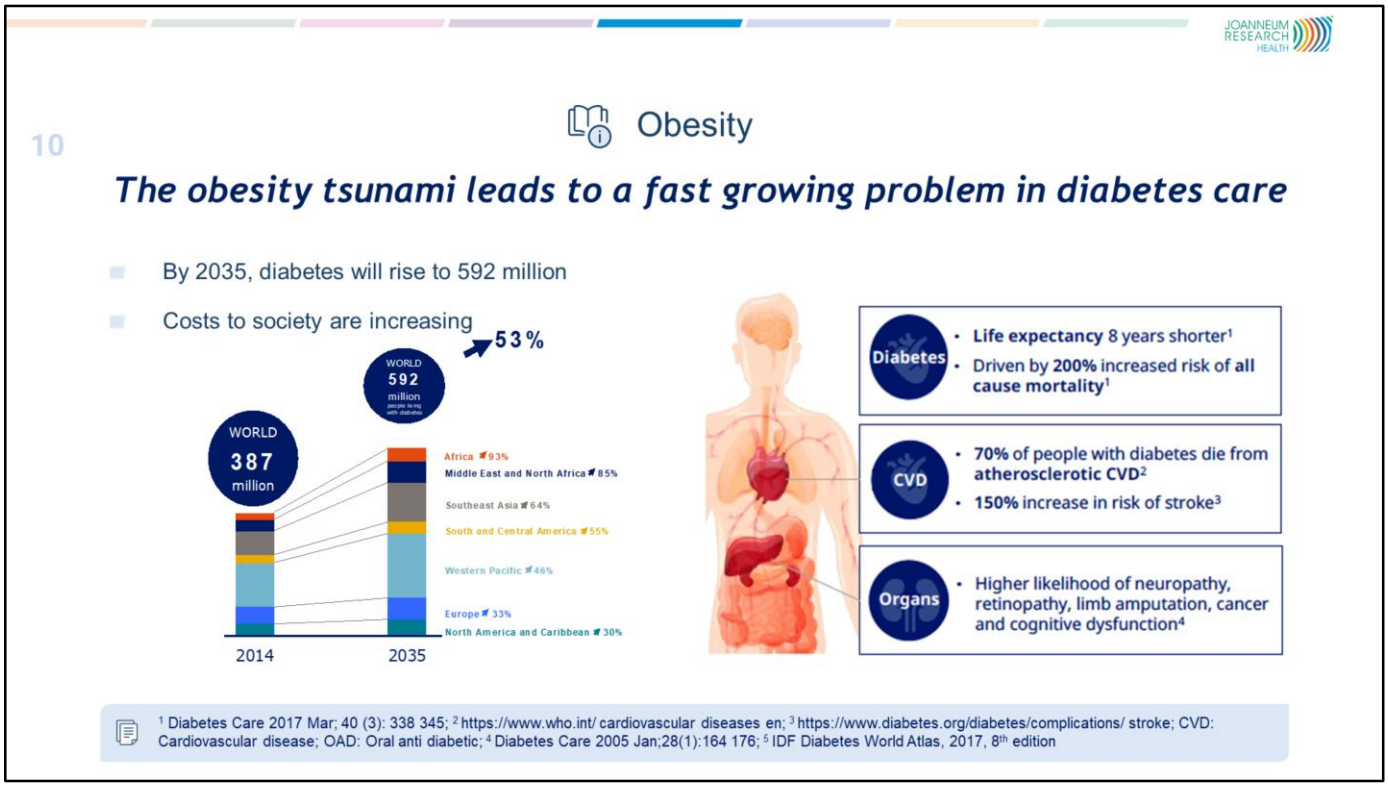
This figure shows the long list of different comorbidities, including a number of pulmonary diseases, gastrointestinal diseases, cardiovascular diseases, etc. and it also shows that the whole body is affected by obesity as a disease. We can group the complications into e.g. mechanical complications that describe all the effects coming from the weight that the body has to bear. Then, and this is the focus of today's webinar, there are metabolic and cardiovascular complications. But of course we have to be aware that there are also psychological complications. And there's even an increased risk of cancer, if you are affected with obesity.



This slide shows the increased risk of those severe and sometimes life threatening comorbidities. On the left side the list includes increased cardiovascular diseases, heart failure, sleep apnoea, osteo-arthritis and diabetes, all of which are interlinked.

The graph in the middle shows that of the 650 million people with obesity only a little more than 10% actually visit a doctor because of obesity and even less have an adequate diagnostics. Only 4% are actively managed and only 2% receive an obesity treatment in terms of a pharmaceutical intervention.

From that perspective, obesity research is really a large field for substantial development in the future. The right panel shows the global healthcare costs that are estimated roughly to be 1 trillion US dollars in 2020 and will increase by 20% in the next five years.



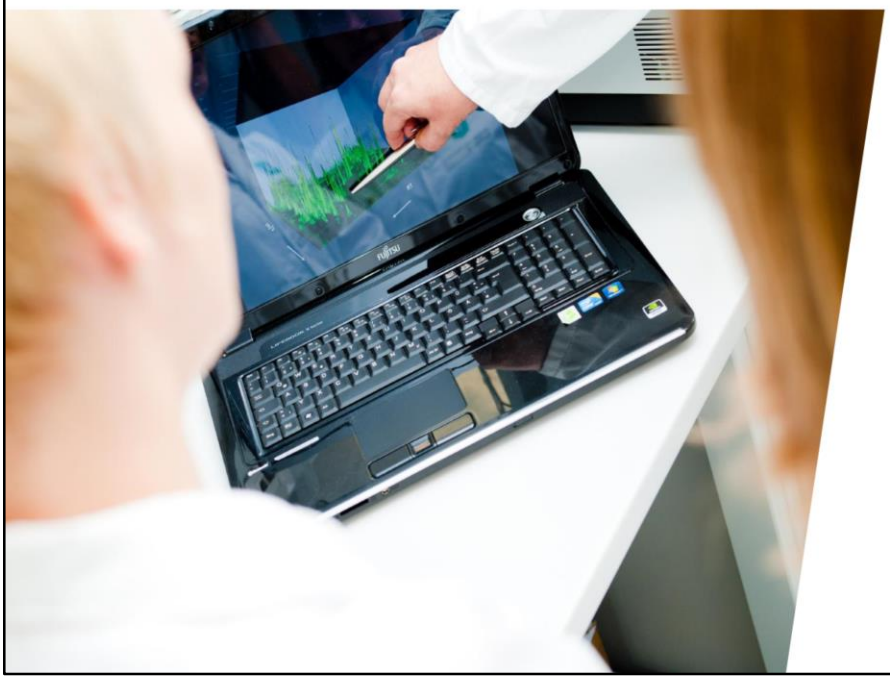
The obesity tsunami unfortunately leads to another tsunami that I call the diabetes tsunami which describes the fastest growing problem in diabetes care.

On the left side there is the increase in diabetes incidence as estimated by the International Diabetes Federation by the year 2035. The increase worldwide is above 50% and every part of the world will be affected leading to an estimated number of roughly 600 million people with diabetes in 2035.

Diabetes has a considerable impact on general health at it reduces life expectancy, roughly by about eight years, and it increases the mortality by 200%. There's a 70% increase of people with diabetes, that suffer from atherosclerotic diseases or cardiovascular diseases, and there is an expected increase in the risk of strokes. There is also a high likelihood that diabetes affects other tissues and organs such as retinopathy, neuropathy, nephropathy, and an increased risk of cancer and other complications.

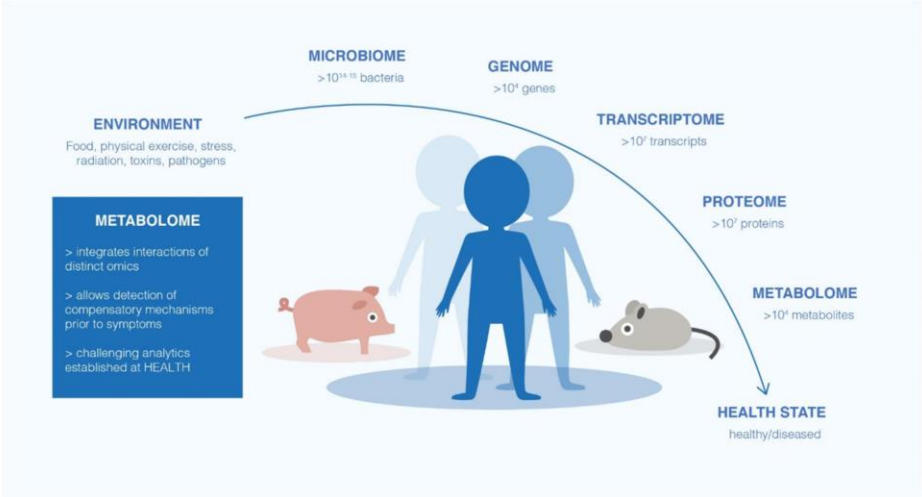
Obesity is a problem but linked with diabetes this is an even bigger problem. Thus

today’s focus is on the use of metabolomics in diabetes and obesity research.



How to use metabolomics in diabetes and obesity research?

Metabolomics

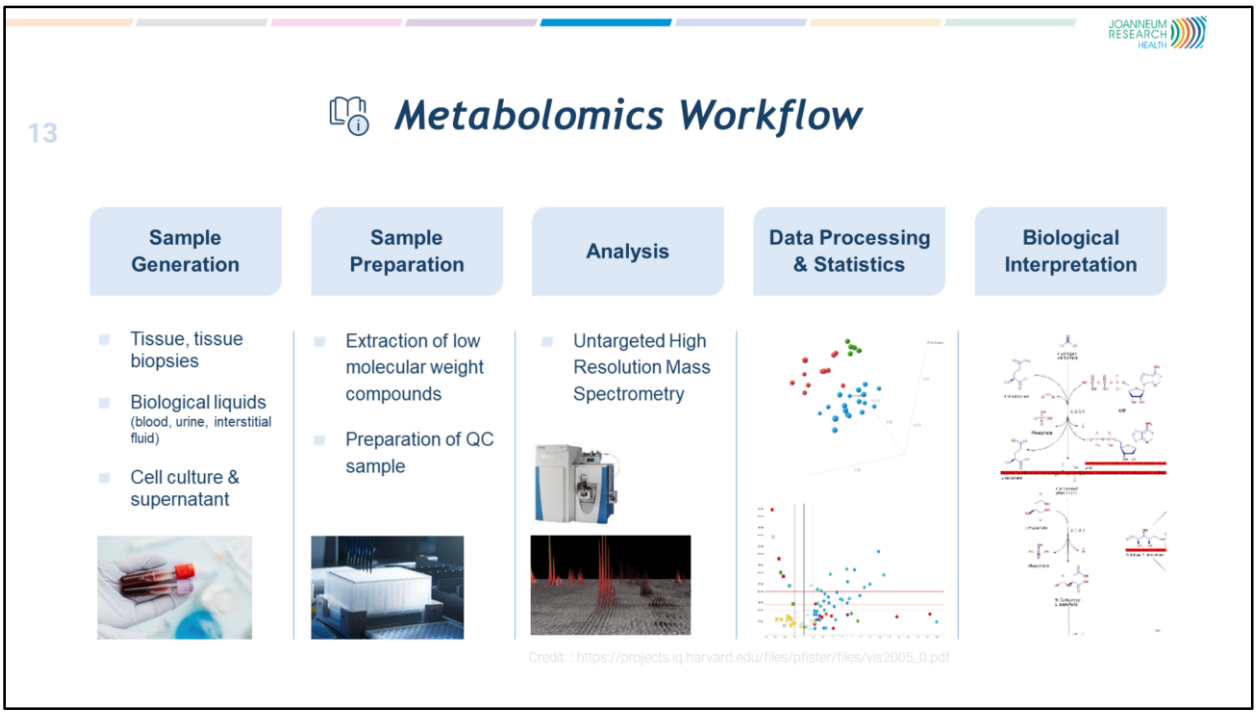


Credit: JOANNEUM RESEARCH

What is the Metabolome? The metabolome represents the complexity of all low molecular compounds in a biological system. Of all biomolecular levels that we can measure (such as the genome, the transcriptome etc.) the metabolome is acknowledged as the closest link to the phenotype.

Because the majority of energy transfer in a biological system occurs through molecular reactions of low molecular weight compounds. Furthermore, all biological macromolecules are composed of building blocks of small molecules.

The status of the metabolome results from the interaction of all biomolecular levels, the genome, the transcriptome, the epigenome, the proteome, and in addition, of various environmental factors. The metabolome is thus a valuable readout of the current status of a biological system and strategically the metabolome is a perfect starting point for hypothesis generation. The metabolome provides an overview - the big picture.



This is the workflow for our untargeted mass spectrometry (MS) based metabolomics platform.

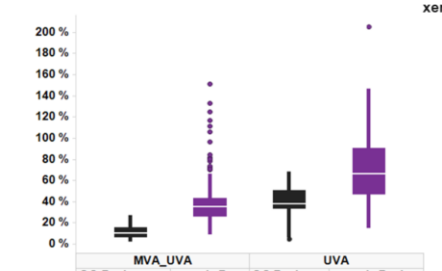
The first step is the metabolite extraction. For blood metabolome analysis we prefer EDTA plasma samples. For EDTA plasma extraction we use cold methanol extraction, where we extract the metabolites overnight in the -80°C freezer. We have also implemented various extraction methods for tissue and cell culture. To monitor the analysis performance during the HILIC-HRMS analysis we generate a pooled quality control sample containing equal aliquots of every study sample. We use a high resolution MS system – the Q Exactive Orbitrap - coupled to hydrophilic interaction chromatography to analyze the samples. In our experience this combination provides a very good coverage of the blood metabolome. With this approach we are able to detect very hydrophilic compounds e.g. amino acids but also e.g. more lipophilic compounds such as phospholipids.

After the LC-MS analysis an elaborate data processing workflow starts containing peak detection, peak alignment through the samples, compound identification and a comprehensive data set quality evaluation by processing the data generated from the quality controls. The quality evaluation provides quality parameters (RSD, drift,

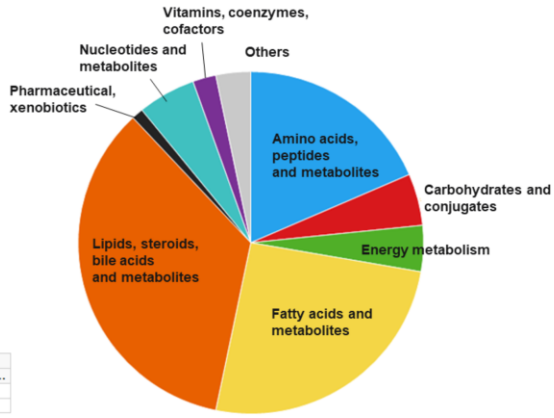
identification quality) for each detected compound. The compounds are grouped in quality levels which qualifies the data for each compound for different statistical processing steps. In a last step biological interpretation by pathway enrichment analysis and pathway mapping are performed.

Metabolomics Quality Report

- Detected known metabolites: >200
- Detected metabolic features: 1500-2000
- Quality controls: Median Multivariate statistics RSD 8-15%
- Mass deviation <5 ppm



	MVA_UVA		UVA	
	QC.Peakarea...	sample.Pe...	QC.Peakar...	sample.Peak...
Count	184	184	41	41
Median	10.4 %	35.2 %	38.1 %	66.4 %



This is an excerpt of a quality report for a give data set. Our workflow always starts with a targeted data analysis. In this step we focus on known metabolites where identification is confirmed by an in-house compound library. This library contains more than 400 compounds (extracellular and intracellular metabolites).

We usually detect more than 200 known metabolites with high quality. The untargeted data processing detects about 1500-2000 features.

The pie graph on the right shows the compound classes covered by the targeted data processing approach. All important metabolite classes are represented.

The box plot shows the relative standard deviation (RSD) of the compounds in the quality controls and in the samples. We usually achieve a median RSD of 8-15% in the high quality metabolites. Biological information is present in the data set if the RSD of the quality control samples are lower than the RSD in the samples. The average mass deviation was below 5 ppm – the mass accuracy expected by the Q Exactive MS.

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Case Study: Alternate Day Fasting

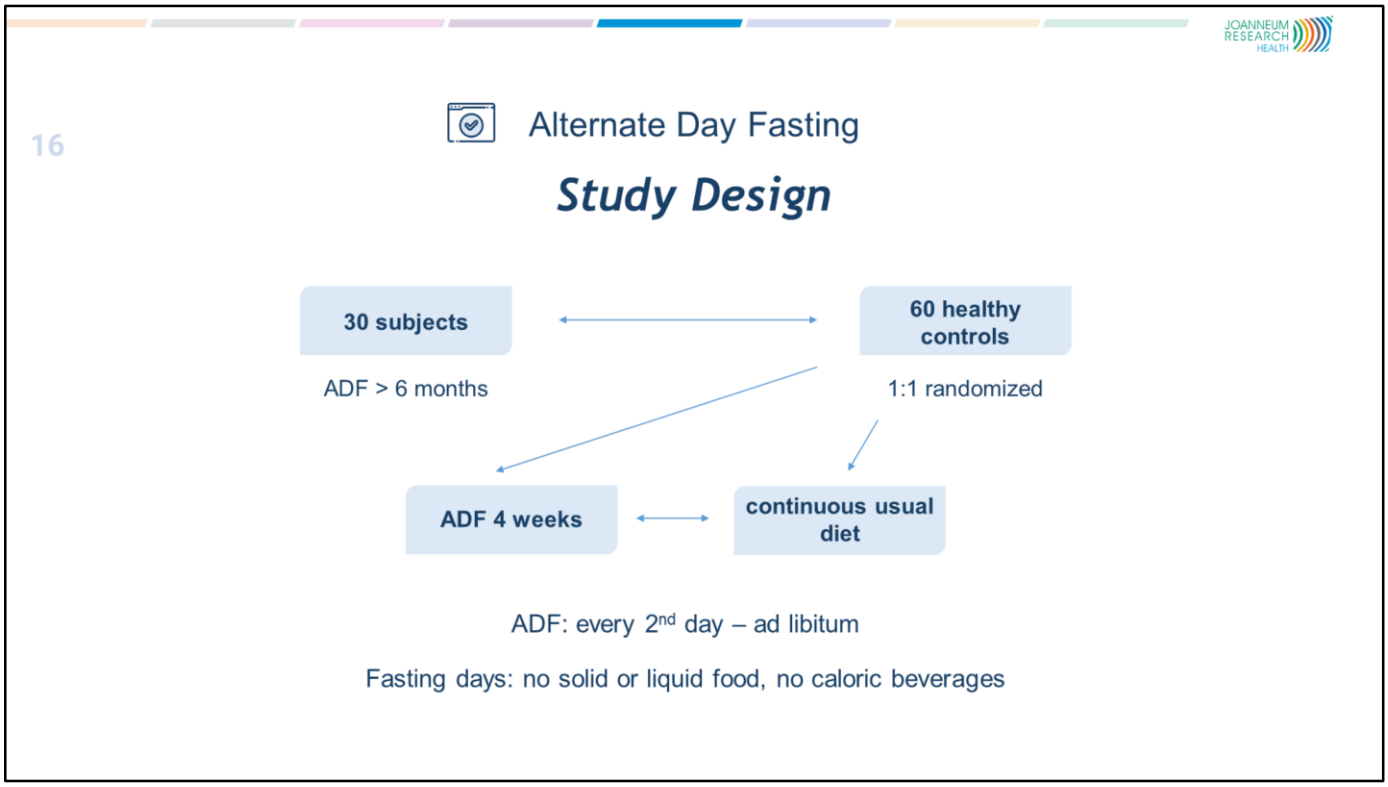
- Can Alternate Day Fasting (ADF) be an alternative to caloric restriction?
- How is metabolism affected by ADF?

Stekovic et al., 2019, Cell Metabolism 30, 462-476 September 3, 2019. https://doi.org/10.1016/j.cmet.2019.07.016

This case study was a collaboration with Thomas Pieber as the clinical lead and Frank Madeo who is a well known researcher in the field of aging with a focus on healthy aging here in Graz.

Up to date all caloric restrictions have led to extended lifespans in all studied species. From single-cell organism, nematodes and fruit flies to rhesus monkeys.

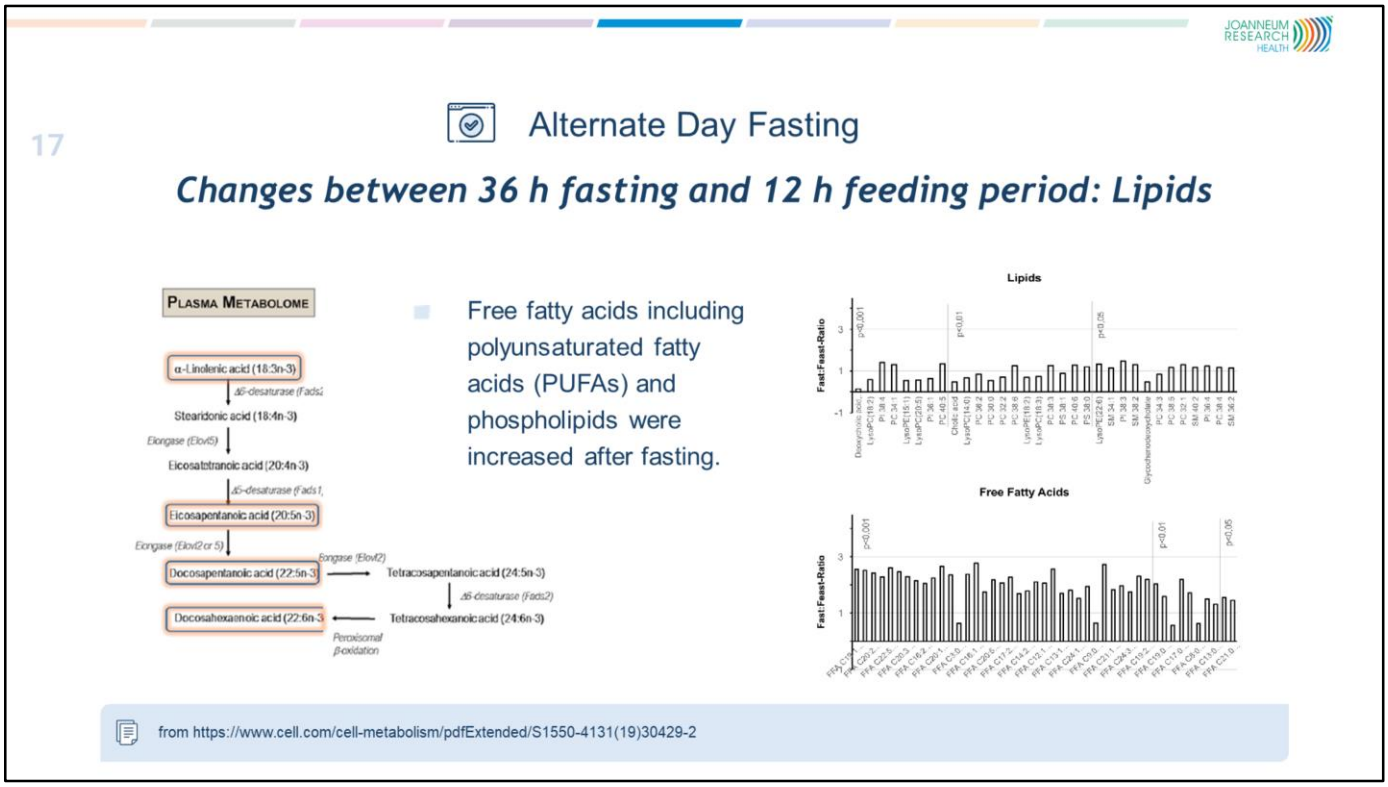
The main question was whether alternate day fasting can lead to similar effects? Currently, alternate day fasting is the latest hype in dietary advice. This means applying alternated periods of fasting and eating. In this study many readouts were generated – today I will focus on the metabolomics readouts.



In this study a specific alternate day fasting protocol also known as 36:12 was used. This means 36 h of fasting (say one day and one night) and 12 h of eating. The first group were 30 healthy people that had already performed alternate day fasting 36:12 for at least 6 months. These 30 subjects were compared with 60 healthy subjects who had not previously performed ADF.

To examine the short-term effects of ADF after 4 weeks, these 60 healthy subjects participated in an RCT and were randomized into a control group of 30 subjects who maintained their usual diet and a second group of 30 subjects who started the ADF protocol. During fasting no solid or liquid food and no caloric beverages were allowed.

The study had many different readouts (for example proteome, hormones, inflammation parameters, bone densitometry and body composition, energy expenditure), for today's webinar I will focus on the metabolomics readouts.



These are the metabolomics results. Overall data show that fasting is a powerful metabolic intervention.

This slide details the results for free fatty acids and lipids.

More than 50 lipid species were at least 20% increased after fasting. Circulating free fatty acids are higher due to activated lipolysis.

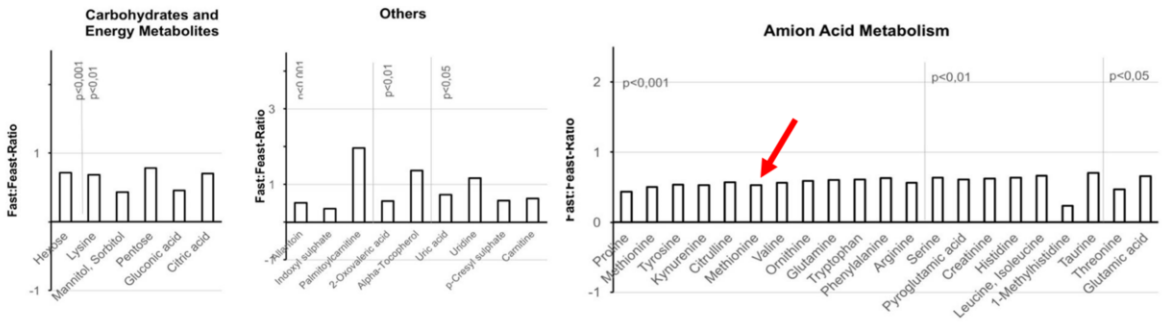
We also found that a broad range of phospholipids and some bile acids were increased after the fasting period.

On the left site the pathway for the metabolic conversion of alpha-linoleic acid to longer polyunsaturated fatty acids (PUFAs) is shown. PUFAs with their known beneficial health effects were significantly increased after 36 h fasting.

Alternate Day Fasting

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:

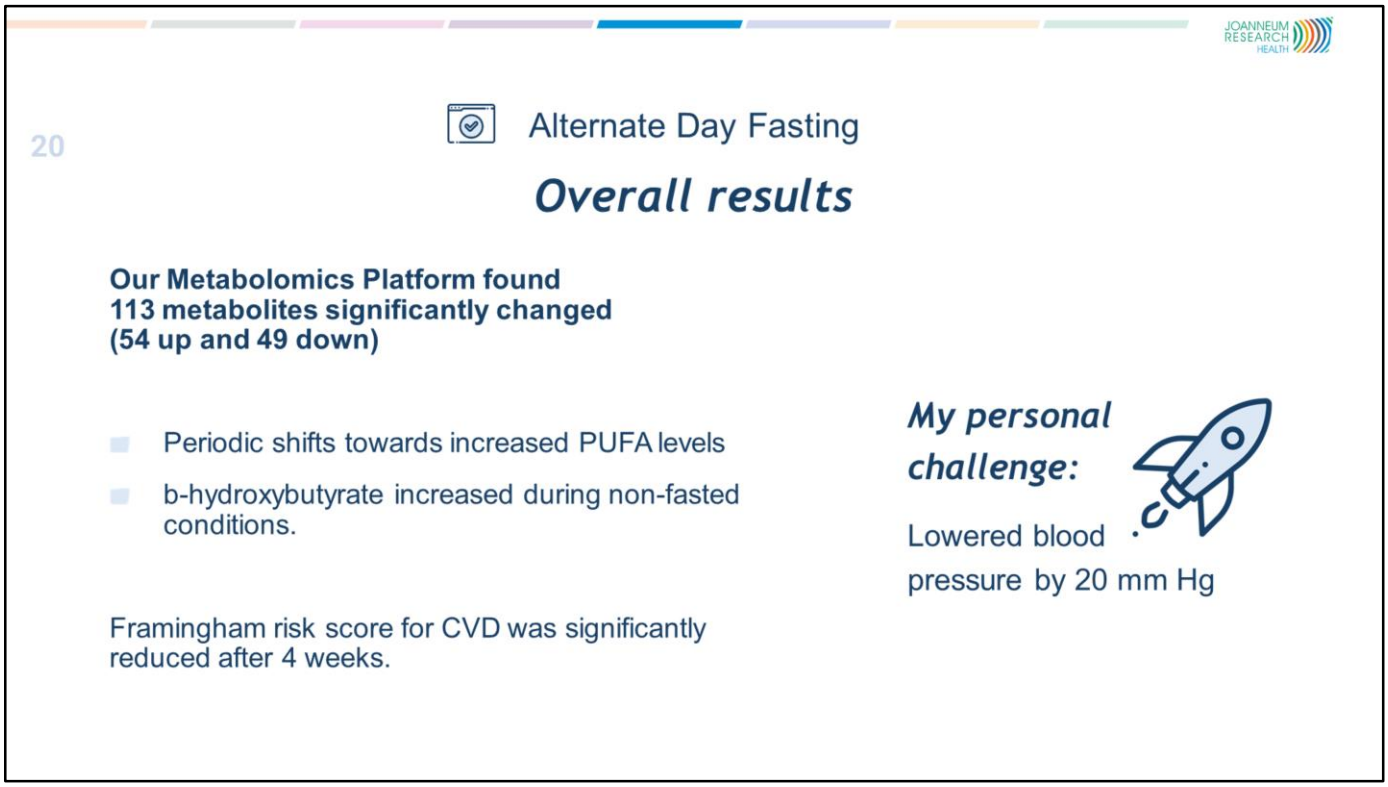


We also found 49 metabolites that were at least 20% decreased after fasting.

Most of these metabolites were amino acids and related metabolites such as ornithine, citrulline and taurine.

Note, low systemic concentrations of amino acids, especially methionine have been shown to be sufficient for lifespan extension in model organism.

A Metabolite Set Enrichment Analysis confirmed a decrease of metabolites in the urea cycle and ammonia recycling pathways and several pathways associated with amino acid metabolism. These changes can be attributed to enhanced lipolysis in adipose tissue coupled to enhanced hepatic capture of amino acids for gluconeogenesis.



Our metabolomics approach was able to detect more than 100 metabolites that were significantly changed after fasting. The study revealed some beneficial effects of alternate day fasting.

Periodic shifts towards increased PUFA levels could be beneficial due to their immunomodulatory and cardioprotective properties. Furthermore, periodically elevated ketone bodies such as beta-hydroxybutyrate might contribute to long-term health span improvements and cardioprotective effects. The Framingham risk score for CVD was significantly reduced after 4 weeks ADF.

Hydroxybutyrate levels were increased also during non-fasted conditions after 4 weeks ADF. Increased beta-hydroxybutyrate levels indirectly reduce blood pressure caused by high salt consumption and improve cerebral blood flow in aging brain.

A small note at the end: I was also able to achieve a small personal success with alternate day fasting. After several weeks of ADF, my blood pressure decreased by 20

mm Hg.

As you can see, our metabolomics approach was able to provide an overall picture of the changes in metabolites following this fasting intervention.

Other Case Studies

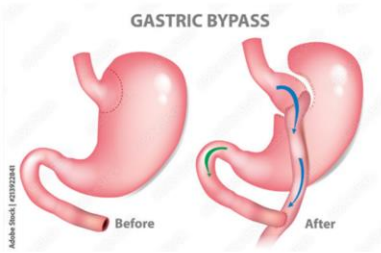
RESEARCH ARTICLE

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

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

1 JOANNEUM RESEARCH Forschungsgesellschaft mbH HEALTH Institute for Biomedicine and Health Sciences, Graz, Austria, **2** Medical University of Graz, Department of Internal Medicine, Division of Endocrinology and Diabetology, Graz, Austria, **3** Institute for Medical Informatics, Statistics and Documentation Medical University of Graz, Graz, Austria, **4** eSwiss Medical & Surgical Center, St. Gallen, Switzerland, **5** CBmed – Center of Biomarker Research in Medicine, Stiftingtalstrasse 5, 8010 Graz, Austria

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

In this study we performed metabolomics analyses before and after bariatric surgery. Bariatric surgery is a very invasive surgical intervention to treat severe obesity.

We were able to detect many differences in the low molecular weight fraction in the blood. The cardiovascular risk associated metabolites valine, alanine, choline, leucine/isoleucine, phenylalanine and tyrosine were significantly decreased after bariatric surgery. Interestingly TMAO, a metabolite related to the gut metabolome and related to cardiovascular risk was significantly elevated after this intervention.

Other Case Studies

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

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

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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

Check for updates

Results overview

- 59 significantly decreased metabolites in Adipocyte Glucocorticoid Receptor Deficient Mice ($GR^{\Delta Ad}$)
- 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

Another interesting preclinical study in adipocyte glucocorticoid receptor deficient mice was performed together with the University of Veterinary Medicine in Vienna.

We were able to show that the basal metabolic signature of these ad libitum-fed mice was significantly different with lower metabolites levels compared to the control group.

The majority of these decreased metabolites were related to fatty acid/lipid metabolism and amino acid metabolites (mainly proteogenic and branched chain amino acids). We concluded that the glucocorticoid receptor in adipocytes exerts a central but divergent role in regulating metabolic homeostasis, depending on the energetic state. The adipocyte glucocorticoid receptor is indispensable for the feeding-fasting transition but also promotes obesity and associated metabolic disorders in fat-fed and aged mice.

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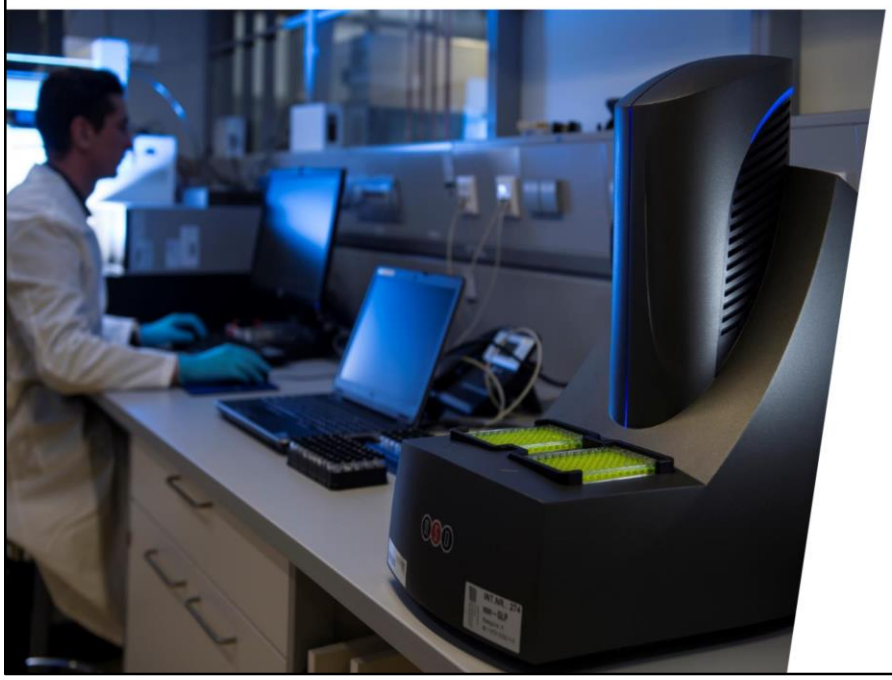
Other Selected Metabolomics Studies @ JOANNEUM RESEARCH

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

These are the references for a few more studies that we have done in this field. We do not only perform studies on diabetes or obesity. We also use our metabolomics platforms for studies in cancer or for biotechnological issues. This demonstrates the broad field of application of metabolomics.

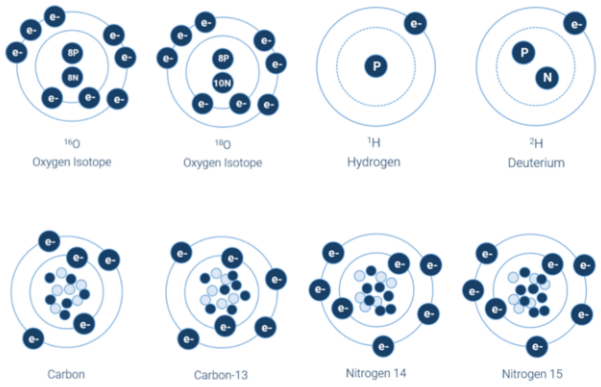


How to use stable isotope tracers in clinical studies?

Stable Isotope Tracers in Metabolic Research

Important Applications

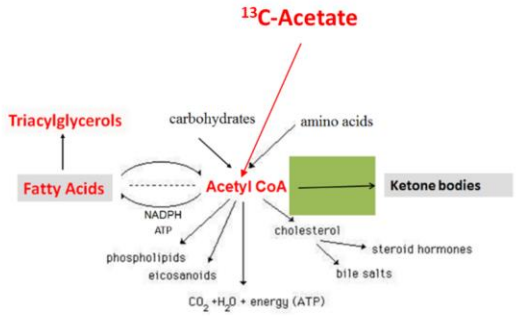
- Metabolite turn-over rates (in-vivo)
- Lipolysis
- Glucose kinetics
- Body water content and total energy expenditure
- Metabolic pathway tracing

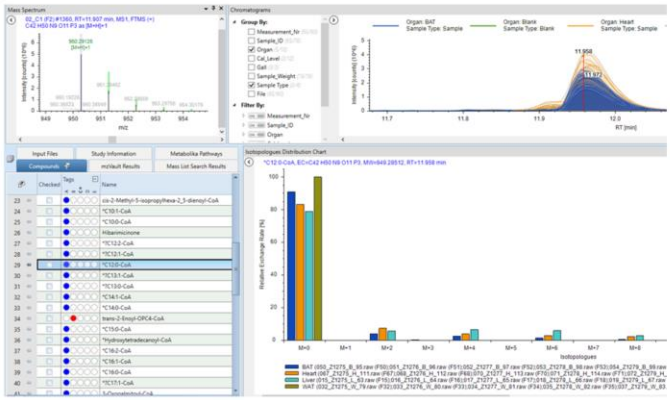


Another interesting method for clinical metabolic research are stable isotope tracers methods. Stable isotopes have the same (or almost the same) chemical properties, but a different atomic mass, which can be distinguished by mass spectrometry.

Different metabolites can be labeled with stable isotopes. With these labeled metabolites, a mass spectrometrically detectable trace can be placed in the living organism. Stable isotope tracers can be used to determine metabolite turn-over rates, lipolysis, glucose kinetics, body water content and total energy expenditure and furthermore to trace metabolic pathways.

Metabolomics Supported by Stable Isotope Labelling

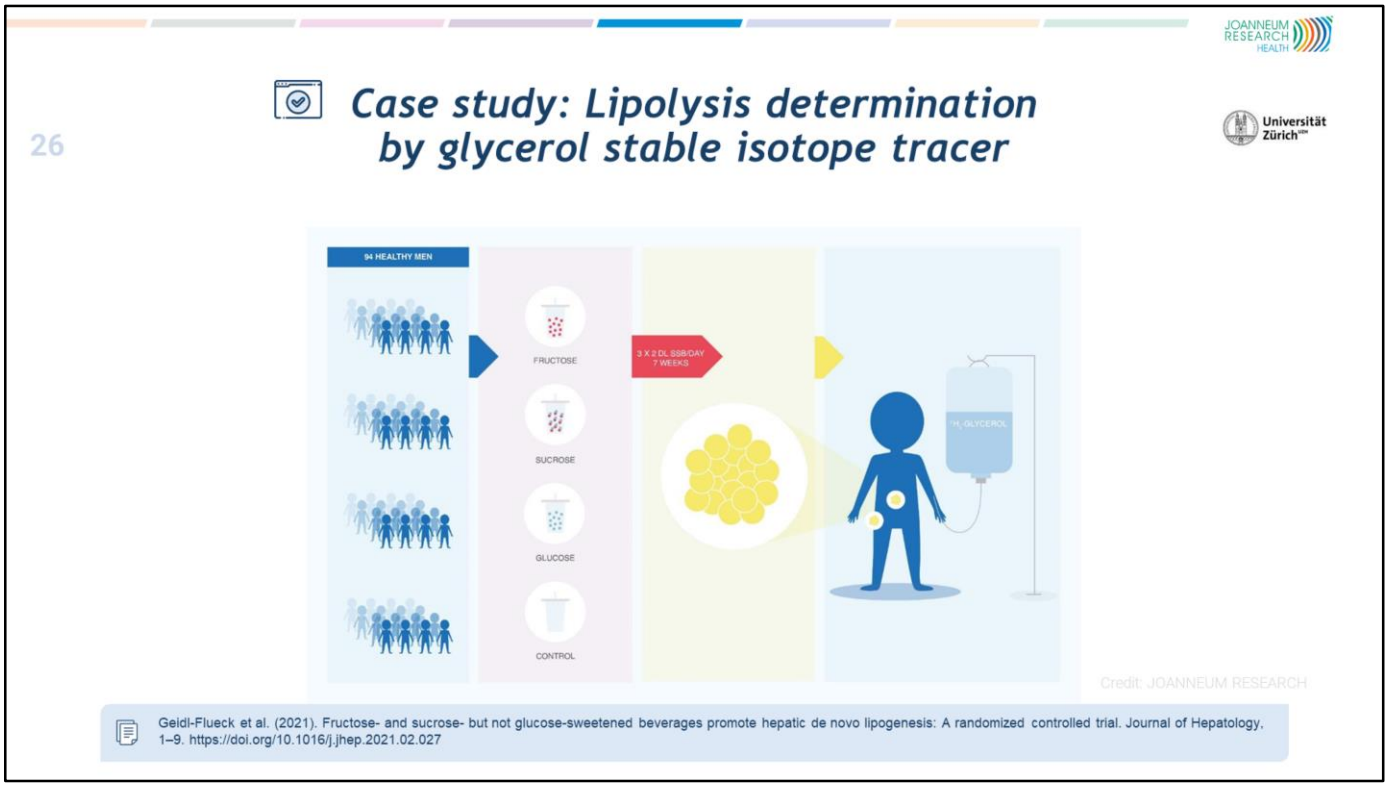




Here is an example how we can trace metabolic pathways by stable isotope labeling. Metabolomics study can be also profit from adding stable isotope labeling.

We performed a study in mice using ^{13}carbon-labeled acetate as labeled substrate. Via acetate the label is incorporated into acetyl-CoA, a central or the central metabolite in the energy metabolism. Via isotopomer analysis we investigated the activity of the different pathways using acetyl-CoA as substrate.

The incorporation of ^{13}C in to C12-0-fatty acid-CoA in different tissues (brown adipose tissue BAT, Heart, Liver and white adipose tissue - WAT) is shown here on the right. The ^{13}C-labels can be found in the same way in the different fatty acid CoAs, confirming the activity of the fatty acid synthesis pathway in the specific tissue.



This case study shows the application of the glycerol tracer to determine peripheral lipolysis to study the effect of caloric beverages on metabolism.

This study was led by the University of Zurich to investigate whether fructose in liquid form in caloric beverages impacts liver metabolism. Fructose is debated to drive the metabolic syndrome or non-alcoholic fatty liver disease. Our contribution was to analyze peripheral lipolysis. 94 healthy men were included in this study. 23 subjects were assigned to the fructose group, 23 to the sucrose group , 24 to the glucose group, and 24 were to the control group. After 4 weeks of sugar sweetened beverage abstinence, individuals started a 7-week intervention with three times daily consumption of a 2 dl of sugar sweetened beverage containing 13.3 g/dl of either fructose, sucrose or glucose with their regular meals, or continued sugar sweetened beverage abstinence.

In week 6 peripheral lipolysis was measured by applying a constant infusion of deuterated glycerol over 4 hours and by enrichment measurements in blood samples.

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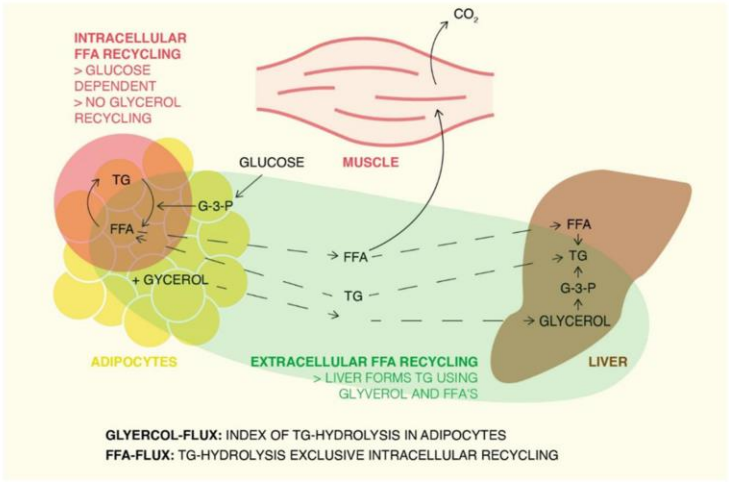
Peripheral Lipolysis - FFA recycling



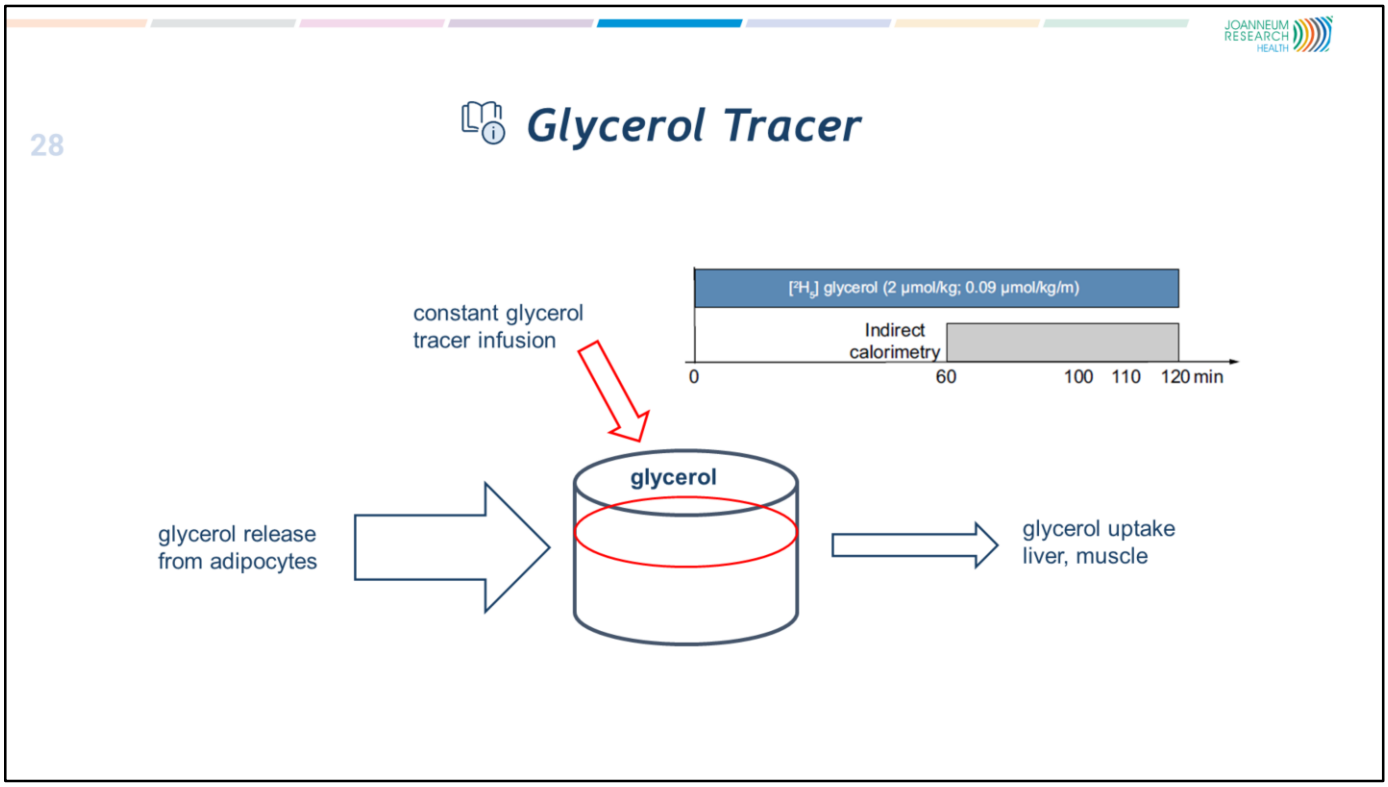
Credit: JOANNEUM RESEARCH

Our contribution was to analyze peripheral lipolysis.

Peripheral lipolysis is defined as the lipolysis in the adipocytes. This part of lipolysis can be analyzed by determining the rate of appearance of glycerol in the blood.

During lipolysis in adipocytes, triglycerides are hydrolyzed by ATGL and HSL to free fatty acids and glycerol. Glycerol and FFAs are released into the blood. Important to note is, that there are two FFA recycling mechanisms. One intracellular in adipocytes, where FFAs are reesterified with glycerol-3-phosphate generated by glycolysis to triglycerides.

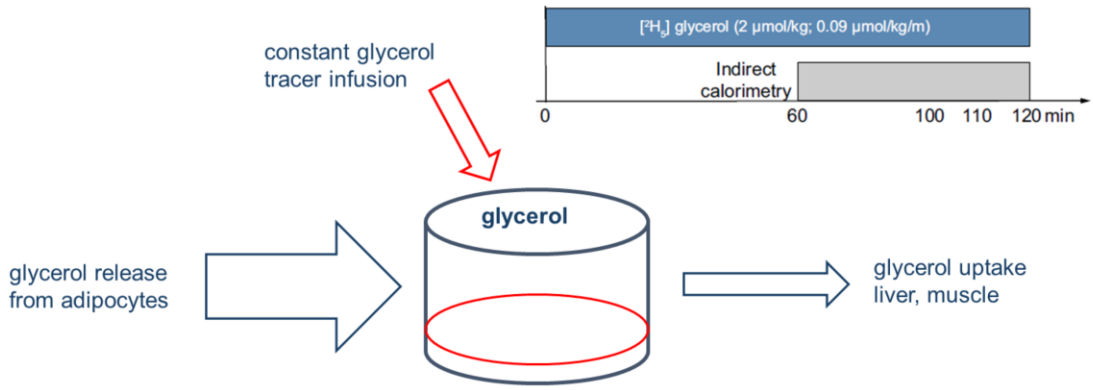
Glycerol from lipolysis can not be used for this reesterification. The second extracellular FFA cycle occurs in the liver, where FFAs and glycerol are esterified to TGs and released to the blood. TGs can be incorporated into the adipocytes. Therefore, the rate of appearance of glycerol is a direct measure of adipocyte lipolysis.



The next two slides show how the rate of appearance of glycerol is determined.

At time 0, a constant infusion of labeled glycerol (0.09 µmol/kg/m in our experiment) begins. The level of labeled glycerol and the ratio of labeled to unlabeled glycerol - the so called tracer to tracee ratio - increases in the blood volume. The reached concentration of labeled glucose in the blood volume depends on the glycerol uptake in the muscles and in the liver. Uptake of labeled and unlabeled glycerol is equal as there is no chemical difference. If lipolysis increases in adipocytes, unlabeled glycerol is released and the ratio of labeled glycerol to unlabeled glycerol decreases. This effect can be used to calculate the rate of appearance by measuring the enrichment of 2H5-glycerol and the concentration of glycerol. We analyzed this by GC/MS using the acetylated derivative of glycerol.

Glycerol Tracer



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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

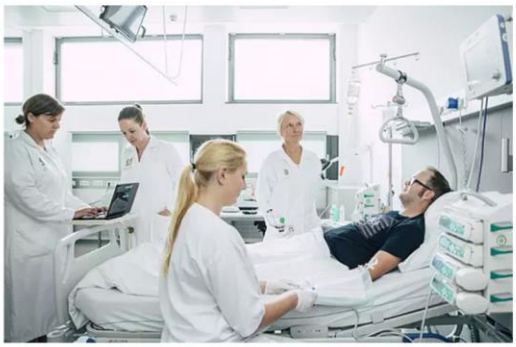
F.....isotope infusion rate: *constant Ib (0.1 µmol)*

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

Coyle et al., 2001, Am J Physiol Endocrinol Metab. 2001;280(3):E391–8. https://doi.org/10.1152/ajpendo.2001.280.3.e391

The rate of appearance is calculated according to Steel. This formula works if we can assume that glycerol is distributed in only one compartment (e.g. blood). As glucose has a more complicated distribution, this approximation does not work. For glucose, at least two compartments (blood, interstitial fluid) contribute significantly to the distribution dynamics.

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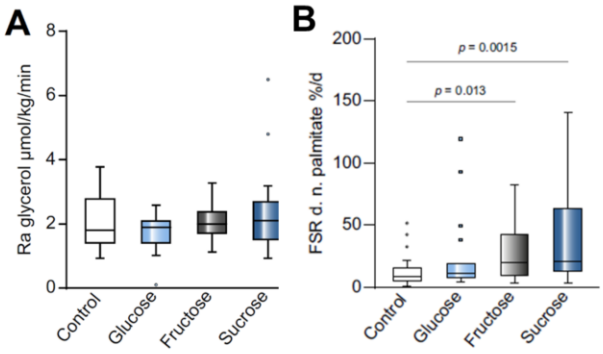
Lipolysis determination by glycerol stable isotope tracer

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.



In the case study we observed no differences in energy intake, peripheral lipolysis, total fatty acids, and fatty acid oxidation. Interestingly, fructose and the glucose/fructose disaccharide sucrose increased hepatic fatty acid synthesis by a factor of two after 7 weeks of consumption. This suggests that the increased basal hepatic fatty acid synthesis is likely the first metabolic change induced by regular fructose-containing sugar-sweetened beverages.

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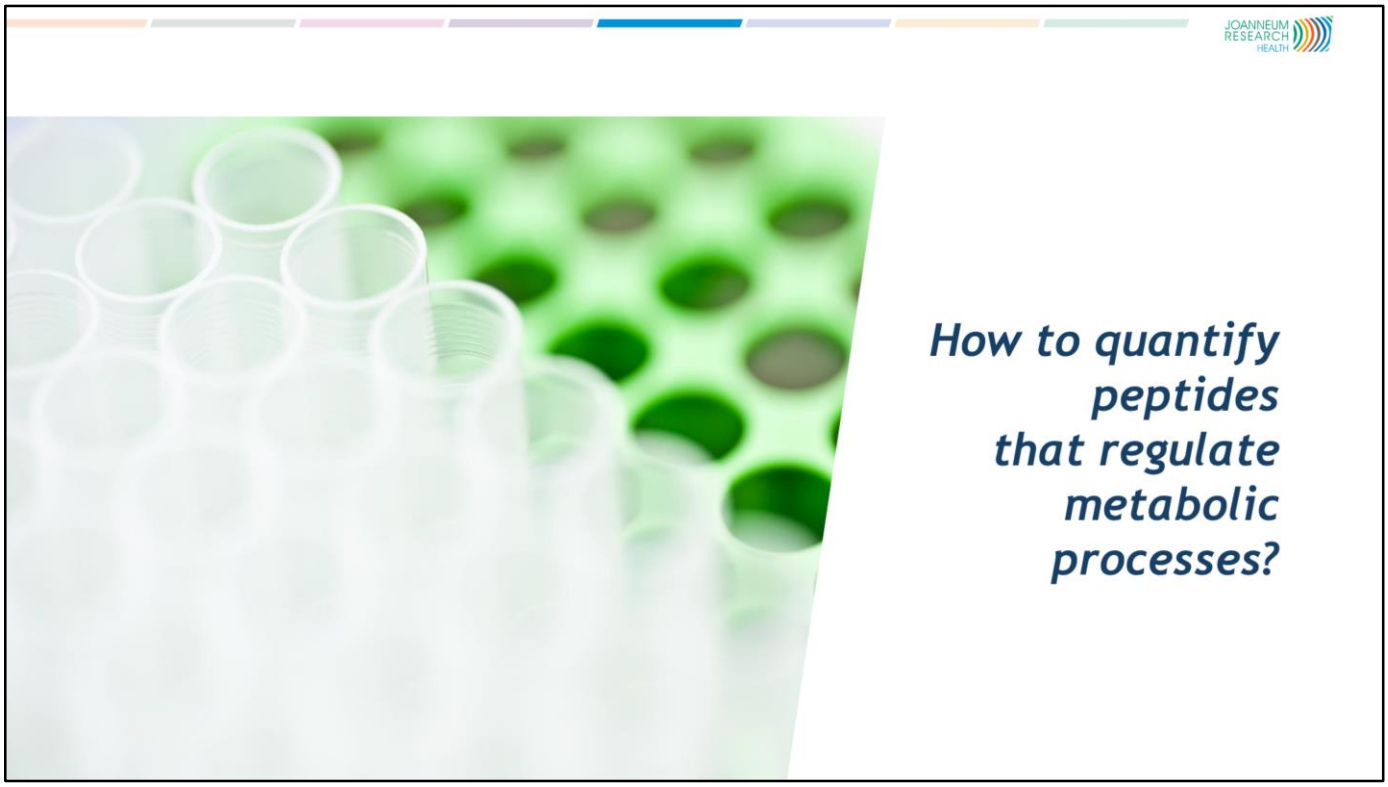
Other Selected Tracer Studies @ JOANNEUM RESEARCH

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: 10.1210/jc.2017-01836
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:10.1111/j.1463-1326.2007.00734.x
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: 10.2337/diacare.28.5.1107
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

Here are some selected clinical tracer studies performed at our institute.

In this fist study we tested endogenous glucose production during hypoglycemia in C-Pep negative and C-Pep positive type 1 diabetes patients.

In the second study we tested SGLT-2 inhibitors and DPP-4 inhibitors on glucagon levels, EGP and lipolysis in type 2 diabetes patients. We used glucose and glycerol tracer in parallel to access EGP and lipolysis in eu-, hyper and hypoglycaemia conditions in humans in-vivo. These studies tested different insulin analogues and their effect on endogenous glucose production.

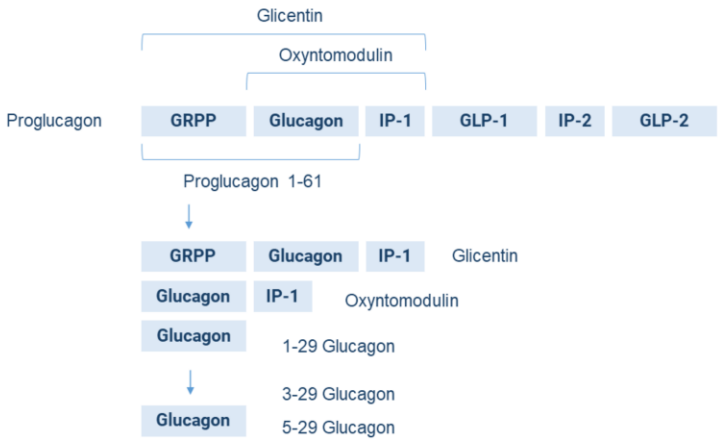


The next topic is also an important part in our analytical research lab.

For clinical studies in metabolic research, reliable, accurate and precise methods are needed to quantify the peptides that are involved in the regulation of metabolic processes. The following slides discuss challenges that are associated with the analysis of glucagon and insulin in clinical studies and present some solutions that we have implemented at Joanneum Research.

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



A challenge in the analysis of endogenous proteins and peptides is that the chosen analytical method needs to distinguish the target analyte from its precursors and also from structurally similar proteins and degradation products. For the quantification of glucagon, this selectivity is a real challenge for most commercially available assays. Most available assays show at least some cross reactivity to the structurally very similar glicentin or to oxyntomodulin (figure on the right). Moreover, glucagon is degraded quickly in-vivo when amino acids are cleaved from the N terminus resulting in a physiological inactive but analytically very similar degradation product.

We thus implemented a highly sensitive ELISA with improved selectivity for intact glucagon.

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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)

*p<0.014 for comparison against healthy volunteers.

Here is an example showing the impact of these selectivity issues on analytical results and on the interpretation of these results. We measured samples of healthy volunteers, type 1 and type 2 diabetes patients with a routinely used radio immunoassay and our selective ELISA method.

The radioimmunoassay (RIA) results were up to 9 times higher which is probably due to the detection of degradation products and other cross-reactive peptides.

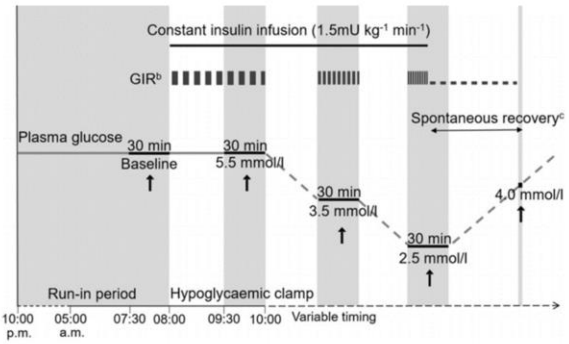
This lack of selectivity masks the significantly higher pathological glucagon secretion in T2D patients compared to the healthy volunteers. The higher glucagon secretion was only detected with our selective ELISA method.

These selectivity issues may have also contributed to the limited understanding of the role of glucagon in diabetes research.

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



Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, et al. Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab. 2018;103(4):1408–17. DOI: 10.1210/jc.2017-01836

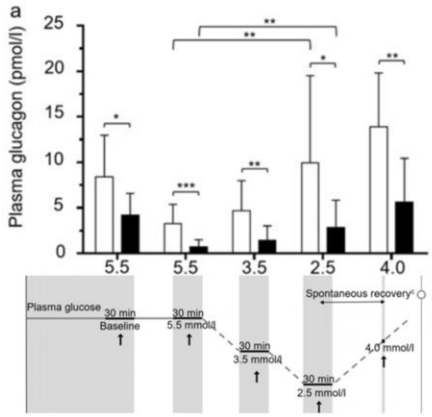
We also used this selective method for glucagon in a clinical study which investigated the response to hypoglycemia in newly diagnosed and long term type 1 diabetes patients.

10 C-peptide positive and 11 C-peptide negative patients underwent a stepwise hypoglycemic clamp induced by a constant insulin infusion with different glucose plateaus.

Glucagon was quantified at each glucose plateau and the endogenous glucose production was determined with the tracer method that was introduced earlier.

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.

Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, et al. Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab. 2018;103(4):1408–17. DOI: 10.1210/jc.2017-01836

In this graph on the left glucagon concentrations are shown at the different glucose plateaus. The white bars indicate C-peptide positive patients and the black bars are the C-peptide negative patients.

Both groups responded to hypoglycaemia: when the glucose concentration decreased, the glucagon secretion increased.

At all glucose plateaus the C-peptide positive patients had higher glucagon secretion compared to the C-peptide negative patients.

And again in all samples, the glucagon concentration was lower than previously reported.

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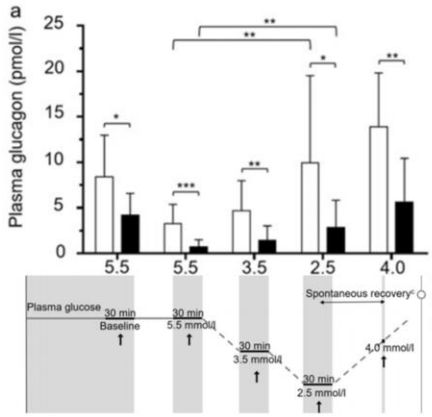
Glucagon in Hypoglycemia

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.

Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, et al. Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab. 2018;103(4):1408–17. DOI: 10.1210/jc.2017-01836

This findings differ relative to previous studies that reported glucagon secretion independent from C-peptide status and glycemic level.

A more selective ELISA could have supported the detection of these physiological differences.

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Quantification of insulin and insulin analogues

- Simultaneous quantification of human insulin and insulin analogues for clinical studies

Here are some results for another peptide: insulin.

Clinical studies that investigate the pharmacokinetics of insulin analogues require specialized analytics to simultaneously determine the concentration of the investigated insulin analogue and human insulin.

Currently a selective assay is commercially available only for one insulin analogue. This makes the analysis of these study samples analytically challenging.

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.

	Human Insulin	Insulin Aspart
LLOQ	3.2 mU/L	6.4 mU/l
ULOQ	201.7 mU/L	101.9 mU/l
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%

To simultaneously determine human insulin and insulin analogues, we are using a combination of a non-selective and a selective ELISA.

With the non-selective insulin ELISA we determine the sum of insulins in the sample and with the selective ELISA we are measuring only human insulin.

The concentration of the insulin analogue is then calculated from these two measurements.

We have established and validated this combined approach for insulin aspart in a GLP compliant process according to EMA guideline on bioanalytical method validation.

The table on the right shows the validation results and shows that we obtained excellent accuracy and precision for human insulin as well as for insulin aspart.

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Quantification of insulin and insulin analogues

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.

Credit: Med Uni Graz

We used this combined method also in a Phase 1 study, sponsored by Arecor a biopharmaceutical company. This study investigated two insulin aspart formulations that are currently on the market and one newly developed formulation.

The study was performed in a randomized, double blind, cross over design with19 type 1 diabetes patients at the Medical University of Graz.

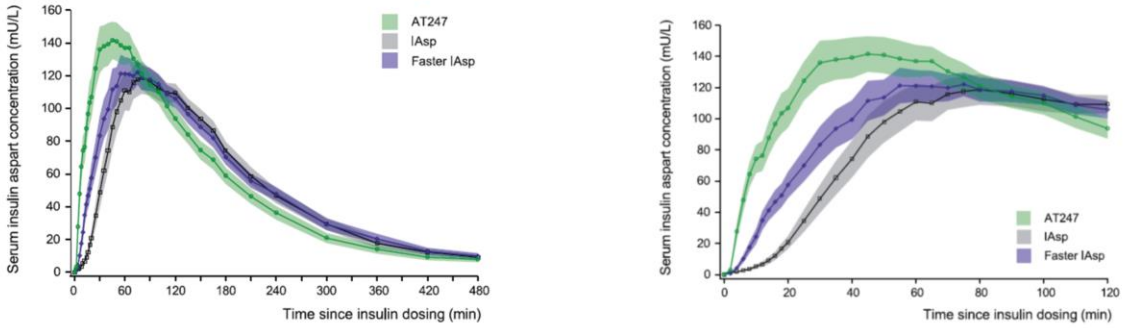
Before the insulin aspart formulation was administered to the patients, blood glucose level was kept constant in an euglycaemic clamp at 5.5 mM by using a glucose and human insulin infusion setup.

Thus, at beginning of the PK profile both insulins were present in the samples and the PK profile had to be background corrected.

PK of insulin formulations

A newly developed formulation showed faster PK

background corrected PK



Svehlikova, E., Mursic, I., Augustin, T., Magnes, C., Gerring, D., Jezek, J., Schwarzenbacher, D., Ratzer, M., Wolf, M., Howell, S., Zakrzewski, L., Urschitz, M., Tschapeller, B., Gatschelhofer, C., Feichtner, F., Lawrence, F., & Pieber, T. R. (2020). Pharmacokinetics and Pharmacodynamics of Three Different Formulations of Insulin Aspart: A Randomized, Double-blind, Crossover Study in Men With Type 1 Diabetes. Diabetes Care, dc201017. doi.org/10.2337/dc20-1017 PMID

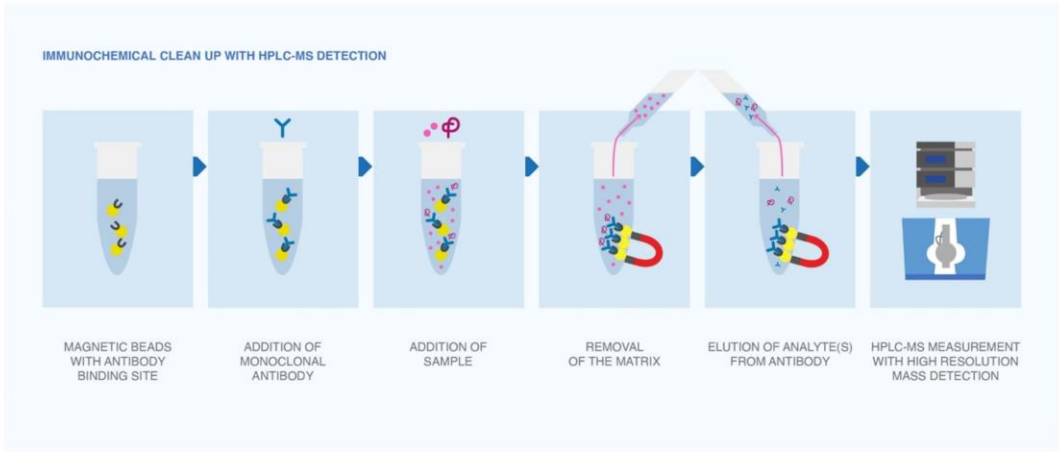
On the left, the PK profiles of the three different formulations are shown over 8 hours, with an increase until minute 90 followed by a steady decrease returning to background levels.

All three formulations have a similar shape but if we look just at the first two hours (on the right) the differences become obvious.

The new formulation, shown in green, had a significantly faster PK in the blood.

Determination of insulin and insulin analogues

Mass spectrometry for insulin analysis



Credit: JOANNEUM RESEARCH

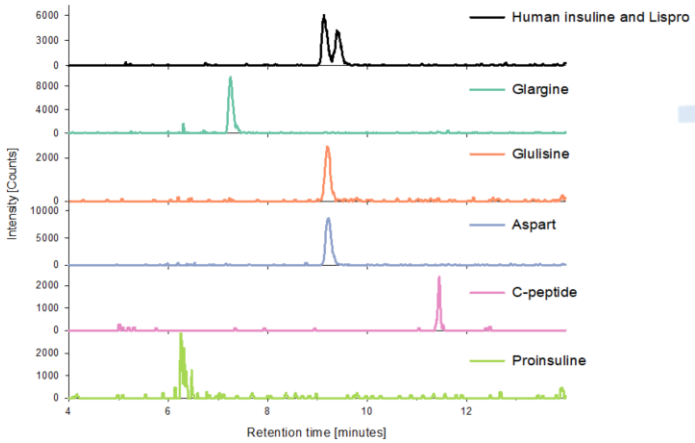
ELISAs are working well for these clinical studies. But what happens if more than two insulin analogues have to be analysed or if other selectivity issues have to be addressed?

An even more selective insulin analysis can be achieved with a combination of an antibody sample cleanup and high performance liquid chromatography that is coupled to high resolution mass spectrometry.

To do so, a monoclonal, but not very selective anti-insulin antibody is attached to magnetic beads. The insulin species in the sample bind to the antibody and can be separated from the matrix of the sample via a magnet. Then the insulin is eluted from the antibody and injected into the HPLC-MS.

Determination of insulin and insulin analogues

Sensitive mass spectrometry for insulin analysis



- 80 pM standard spiked to insulin free serum

We tested this combination of antibody cleanup and HPLC-MS analysis with several different insulin species.

This chromatogram shows a 80 pM mixed standard in insulin free serum. In the first line (black) you can see human insulin and insulin lispro. Both insulins have the same molecular mass and they only differ in the position of two amino acids.

In the next line (turquoise) you can see insulin glargine that has a different molecular mass and a different retention time. Insulin glulisine (orange) and insulin aspart (blue) have the same retention time but their molecular mass allows differentiation.

Also with this method the precursor proinsulin and C-peptide can easily be determined at the same time.

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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