

Asprosin dynamics relating to serum Glucose levels under controlled alteration

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- **Title of the study**

Asprosin dynamics relating to serum Glucose levels under controlled alteration

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3. Summary

The Pilot study entitled „ Asprosin Dynamics relating to serum Glucose levels under controlled alterations“ is designed to date the asprosin kinetics at various metabolic states relating to serum glucose and the correlation between asprosin and the known glucose-regulating hormones.

Asprosin is the C-terminal cleavage product of profibrillin. This new hormone is encoded by FBN1 Gen (Amino acid residues 2732-2871, molecular weight 30 kDa), which also encodes fibrillin. The hormone was initially discovered during the analysis of the genome of patients with the extremely rare Wiedemann- Rautenstrauch - syndrome (Romere C. et al 2016 Cell). According to the data so far, asprosin is a fast- induced protein hormone that acts on the liver through cell membrane receptors, where it activates the G protein cAMP- PKA pathway, leading to a rapid release of glucose into the circulation and to compensatory insulin production. The above seems to match the constellation found in overweight type 2 diabetes patients or patients with metabolic syndrome ("insulin resistance"). Diabetic mice were able to normalize their glucose and insulin levels by asprosin-binding antibodies. According to a recent study (Romere C. et al 2016 Cell), asprosin had no influence on the serum concentration of glucagon, epinephrine, nor- epinephrine and cortisol in mice. In humans it is known that asprosin increases during fasting.

The liver- related glucose release into the blood circulation is crucial for the brain function and overall survival during fasting. In addition, a compensatory rise in asprosin is expected during a hypoglycemic episode. In this pilot study, the asprosin concentration is measured both in participants with type 1 Diabetes mellitus, with or without hypoglycemia unawareness during a hypoglycemic phase. These relevant measurements are compared in the two subgroups, consisting of 5 persons each.

By correlating asprosin values with other regulating hormones, there is hope to better understand the pathomechanism of hypoglycemia unawareness. Recently discovered is that the recombinant asprosin administration in mice allows both the blood glucose and the insulin to rise. It is therefore very likely that further studying of asprosin could provide new insights into the (patho-) physiology of the intermediary metabolism disorder.

According to the above, the following hypotheses arise:

- Asprosin, as fast- induced protein hormone, increases serum levels in type 1 diabetics with or without diabetes late- complications during a hypoglycemic phase.
- Asprosin increases serum levels more significantly in diabetics without hypoglycemia unawareness compared to diabetics with hypoglycemia unawareness.
- There is probably no correlation between asprosin and the previous known regulating hormones.

In order to investigate these hypotheses, study participants will be initially profoundly examined in our Clinical Study Center regarding both their glycemic metabolic responses and the clinical findings relating to their possible micro- and macrovascular complications. In addition to this quality of life, well-being, depression and neuropathic pain is going to be taken into account.

This is to be the first study of its kind, in which plasma levels of asprosin is determined and Type 1 diabetics with and without hypoglycemia unawareness are thoroughly examined in order to identify possible differences and similarities for the better determination of the new hormone utilizing the framework of hyperinsulinemic Clamp- Tests. The intention behind is to better understand the (patho-) physiology of the hypoglycaemia unawareness, as well as to better characterize the physiological properties of asprosin.

4. Scientific background and state of the art

The number of treated type 2 diabetic patients in Germany is an estimated 6 million (Rathmann et al. 2010). However, this number underestimates the true scale of the disease, since, as in most Western-European countries, the prevalence of undiagnosed patients is high (Rathmann et al. 2010). Epidemiologic studies undermine that type 2 diabetes according to the current definition increasingly begins in mid-adulthood (Rathmann et al. 2010). Chronic progress of the disease and the need for ongoing treatment mean a huge burden for the patients. **Especially diabetes- associated late complications cause a significant reduction in life- quality and an increase in mortality (Roglic et al. 2005) and should therefore become the target of future therapeutic efforts.** Complications manifest as macroangiopathy in the sense of coronary artery disease, peripheral artery disease, and cerebrovascular insufficiency. Moreover, as a consequence of microangiopathy, diabetic nephropathy, neuropathy, and retinopathy can occur, leading to renal insufficiency, loss of eyesight, or diabetic foot syndrome as well as the severe acute complication of hypoglycemia unawareness (Schleicher et al. 2001).

Current therapeutic strategies focus on treatment of hyperglycemia (UKPDS, ACCORD, ADVANCE etc). However, under closer observation, HbA1c and therefore glucose metabolism are insufficient surrogate markers for the development of relevant micro- and macrovascular complications. **This means, therefore, that the development of diabetes mellitus and clinically relevant complications especially the hypoglycemia unawareness are inadequately understood.**

The mechanisms leading to this disorder of glucose-insulin metabolism are still not fully clarified. The discovery of the hormone asprosin from colleagues in Houston, USA, could now help us to a new point of view concerning diabetes mellitus (Romere C. et al 2016 Cell). These American scientists originally analyzed the genome of patients with Weidemann- Rautenstrauch syndrome. This is a rare progeroid disorder, in which children appear to age rapidly. The researchers discovered through genome analysis that these patients lack an unknown hormone. They called it "asprosin", from the Greek word "aspros" meaning white.

Patients suffering with progeroid disorder lack subcutaneous white adipose tissue, resulting to lipodystrophy. Further investigations have shown that asprosin is mainly secreted by white adipose tissue, especially during fasting, before reaching the liver via blood- circulation. It there adapts itself to specified receptors and through a procedure including cAMP pathways leads to further glucose- release in the releases glucose into the blood- circulation.

People with Weidemann's Rautenstrauch syndrome exhibit particularly low insulin concentrations, apparently because of this Asprosin deficiency. It was as a result deduced that asprosin is a fasting-induced protein hormone.

The authors recognized that asprosin increases both blood glucose and insulin in mice. However, it did not affect the serum concentration of glucagon, epinephrine, norepinephrine and cortisone in mice.

People with insulin resistance show elevated asprosin levels, as the researchers of the above study noticed. If the recently discovered hormone is suitable as an alternative treatment of type 2 diabetes or a tool for insulin resistance screening awaits still to be answered, as further clinical studies are requested. Pharmacological approaches, such as specified antibodies, targeting Asprosin-inhibition, could reduce the serum glucose and improve insulin sensitivity, according to the current study. In mice with diabetes, the authors succeeded in neutralising the Asprosin's effect using recombinant antibodies, leading to serum glucose and insulin levels reduction. After an injection of recombinant Asprosin the above levels were accordingly elevated.

The situation in newly-diagnosed type 1 diabetics without insulin resistance is similarly undecided. Incidence of type 1 diabetes increases continually (Ehehalt et al. 2012), and due to manifestation earlier in life, the risk for diabetic complications and reduction in quality of life is even more pronounced.

As far as we know, the pathogenesis of type 1 diabetes is an autoimmune destruction of insulin-secreting beta-cells in the pancreas, resulting in absolute insulin deficiency. From our own studies, we know that diabetic complications can be found in rare cases of type 1 diabetics shortly after first diagnosis. As in type 2 diabetes, patients with good blood glucose control (HbA1c 6-7%) can develop diabetic complications over time, whereas some patients with insufficient blood glucose control over long time-periods never develop late diabetic complications (clinical observation). **Therefore, pathogenesis of diabetic complications does not seem to be entirely dependent on glucose metabolism.**

As a result, the only similarity between the two diabetes types seems to be the name „diabetes mellitus“, which only describes a symptom.

In addition to the aforementioned pathomechanisms in this context, in both type 1 and type 2 diabetics, polygenetic factors, environmental-, nutritional- and lifestyle factors, which are supposed to be associated with the development of diabetic complications, are being discussed. These associations with the development of diabetic complications are being examined in the

German Diabetes Study (Deutsche Diabetes Studie, DDS), an ongoing multicenter observational study (in Heidelberg: ethics number **S-232/2013**).

A severe acute complication, which affects predominantly type 1 diabetics (at least a quarter of all type-1 diabetics, Gerich et al., 1991), but also type 2 diabetics with low C-peptide values, is the hypoglycemia unawareness and the associated autonomic failure ("Hypoglycemia Associated Autonomic Failure", HAAF). Reduced glucose serum concentration usually initiates a specific series of responses (Schwarz et al., 1987, Mitrakou et al., 1991, Fanelli et al. 1998). The human organism itself is protected against hypoglycaemia through a physiological mechanism involving the activation of the autonomic nervous system or hormone related counter-regulation. The function of the brain is directly dependent on the glucose supply, because it can neither accumulate it in sufficient quantities nor produce it itself. A reduced insulin release is the first step against hypoglycemia (Cryer 1997). The secretion of regulating hormones such as glucagon (stimulates hepatic glucose production), adrenaline (stimulates hepatic and renal glucose production and restricts the use of glucose by other tissues other than the brain), cortisone and growth hormone (both support glucose production) increase as soon as the glucose level is below the corresponding glycemic threshold (Cryer 1997). In healthy persons, insulin secretion is reduced by a glucose level of about 80-85 mg / dl (4.4-4.7 mmol / l). In the case of values between 65 and 70 mg / dl (3.6-3.9 mmol / l), an increased release of counter-regulatory hormones begins (Schwarz et al 1987, Mitrakou et al., 1991, Fanelli et al. 1998). Normally, most patients will be aware of the first hypoglycaemic symptoms in glucose levels between 50 and 55 mg / dl (2.8-3.1 mmol / l). Cognitive dysfunction occurs by levels below 50 mg / dl (<2.8 mmol / l). A loss of senses is usually expected only by values below 35 mg / dl (1.9 mmol / l) (Schwarz et al., 1987, Mitrakou et al., 1991). Glucose serum levels below 20 mg / dl (1.1 mmol / l) lead to cerebral seizures or coma.

To optimize therapeutic strategies and to better understand and avoid secondary diseases and acute complications such as hypoglycemia unawareness, it would possibly be of great importance to study the new hormone "asprosin". Being a fast-induced plasma glycoprotein measuring or altering Asprosin levels could further help develop individualized prevention and therapy.

In this pilot study, five people with and five without hypoglycemia unawareness will be recruited and comprehensively phenotyped. The participants will be type 1 diabetics with a BMI between 25 and 35 kg / m² and 18 to 75 years of age. An extensive phenotyping of the participants will be performed, which will bring important new data on type 1 diabetes and diabetes related late complications. What takes place is the characterizing of the participants regarding their Asprosin

levels when fasting and after glucose or insulin administration (hyperinsulinemic Clamp test) as well as of the diabetic late complications even in the subgroups that form. Eventually, the correlation of the asprosin serum concentration against the other regulating hormones such as glucagon, catecholamine and cortisol will be thoroughly evaluated. A platform for discussion is created in this way, one that can still not be reached through current studies.

This is to be the first study of its kind, in which plasma levels of asprosin is determined and Type 1 diabetics with and without hypoglycemia unawareness are thoroughly examined in order to identify possible differences and similarities for the better determination of the new hormone utilizing the framework of hyperinsulinemic Clamp- Tests. The intention behind is to better understand the (patho-) physiology of the hypoglycaemia unawareness, as well as to better characterize the physiological properties of asprosin.

5. Aims and hypotheses of the study

The following hypotheses shall be studied:

- Asprosin, as fast- induced protein hormone, increases serum levels in type 1 diabetics with or without diabetes late- complications during a hypoglycemic phase.
- Asprosin increases serum levels more significantly in diabetics without hypoglycemia unawareness compared to diabetics with hypoglycemia unawareness.
- There is probably no correlation between asprosin and the previous known regulating hormones.

In order to investigate these hypotheses, study participants will be initially profoundly examined in our Clinical Study Center regarding both their glycemic metabolic responses and the clinical findings relating to their possible micro- and macrovascular complications. In addition to this quality of life, well-being, depression and neuropathic pain is going to be taken into account.

To investigate these hypotheses, associations of „diabetic“ micro- and macrovascular complications with different metabolic pathways, especially glycolysis, insulin resistance, impairment of insulin secretion, psychosocial factors (depression, quality of life), cognitive

parameters, as well as clinical and laboratory parameters will be examined during basic testing in ten persons with diabetes type 1, five persons with and five without hypoglycemia unawareness. Asprosin levels were then determined during fasting periods as well as after glucose and insulin administration (hyperinsulinemic clamptest). The determination of glucagon, catecholamines and cortisol takes place during the execution as well as at the end of the clamp test and are being correlated to Asprosin values.

The findings may be crucial to understanding the pathophysiology of hypoglycemia unawareness and the development of further prevention methods.

Basic testing/cross-sectional analyses:

- Do the participants already manifest diabetes late complications?
- Do Asprosin serum levels increase after 12 hours of fasting in all participants? Is the fasting- Asprosin level higher in diabetics with insulin resistance compared to diabetics without insulin resistance?
- Are the Asprosin values after glucose administration in type 1 diabetics with or without hypoglycemia comparable unawareness and were they indicative of a comparable pathogenesis?
- How do Asprosin levels alter after insulin administration in the different subgroups during a phase of lower glucose serum levels (target range 60-70 mg / dl)? Could a correlation of the asprosin concentration to the known regulating hormones eventually exist?

6. Study design and examinations, Method

It is a clinical pilot study, which will be launched mono-centric. The following examinations are included in study, which are separated into different modules as presented in the following passage.

Recruitment of study participants will take place on medical wards and in outpatient facilities of the University Hospital Heidelberg. Moreover, there will be informative events at the University Hospital Heidelberg where study participation will be offered, and our press office will be informed and will release articles in the local press to raise attention to the study. According to experience, word of mouth will be spread as well. The participants will be ten persons with diabetes type 1, five persons with and five without hypoglycemia unawareness.

People interested in study participation can contact the Clinical Study Center via phone or e-mail. Written information will be sent by post or handed out directly. At a separate appointment, oral and written informed consent will be obtained by a study doctor, so that study participants have enough time to think.

Basic testing will take one day. On the first day are planned various non-invasive clinical routine examinations. Study participants will be invited between 10 and 12 a.m. starving. The basic tests take three to four hours, depending on the number of study participants invited on that day. Quantitative sensor testing takes approximately 90 minutes of additional time, it will be take place on the first day too. On a second day a hyperinsulinemic clamp examination is performed, which will be scheduled separately, within four weeks of basic testing.

The clamp lasts about 5 hours including the follow-up time. Initialy blood is drawn in order to measure of plasma glucose, Asprosin, insulin, C-peptide, adrenaline, norepinephrine, glucagon and cortisone concentrations, and twice for the biobank (at the beginning and end of the test) and for determining the values of the routine lab testing will be performed. In addition, a urine examination takes place. All tests will be performed according to SOPs by trained personnel.

6.1 Study examinations

The study examinations will be organized in modules.

All substances, methods, and medical products used in the study are approved for the use in humans and will be used according to approval.

Module 1: Medical history (questionnaire) and clinical basic testing (mandatory)

Included will be known diseases, blood glucose control, present diabetic complications, medication, allergies, size, weight, and hip/waist-ratio.

Module 2: Blood and urine testing (mandatory)

- **Basic laboratory testing**

- Total blood count (Leucocytes, red blood cells, platelets, hemoglobin, hematocrit, median corpuscular hemoglobin [MCH], median corpuscular volume [MCV], median corpuscular hemoglobin concentration [MCHC])
- Clinical chemistry: Sodium, potassium, calcium, magnesium, phosphate, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, Lp(a), creatinine,

suPAR, calculated GFR, GOT/AST, GPT/ALT, GGT, AP, cholinesterase, hsCRP, total bilirubin, BUN, uric acid, ferritin, transferrin, transferrin saturation, iron (Fe), total protein, albumin

- Urine analysis: urine status, albumin, protein
- **Hormones, diabetes, glucose**
 - Fasting glucose, HbA1c, TSH, Parathormon
- **Samples for biobank**
 - Plasma for metabolomics/biomarkers
Collection of different stabilized plasma samples (Li-heparin, EDTA plasma, PBMC isolation, whole blood, citrate blood, serum, urine) at a fasting state for basic characterization, as well as EDTA plasma during interventional examinations (oGTT, clamp) to analyze different biomarkers, which are linked to the development of late diabetic complications. Collected material will be stored for up to 5 years and eliminated afterwards in case it was not needed for analyses.

With the participation in the clamp study, necessary total blood volume is 270 ml.

Module 3: insulin resistance module and asprosin cinetics

- **Euglycemic hyperinsulinemic clamp study**

It is a standard scientific test for the determination tissue sensitivity to insulin. In this study the asprosin kinetic in various hyperglycemic and euglycemic phases as well as the correlation between asprosin levels to the other known regulating hormones (adrenaline, norepinephrine, glucagon and cortisone) will be investigated. During the hyperglycemic phase, as well as at the end of the clamp test, the mechanical pain sensitivity (MPS) and the mechanical allodynia in the case of moving stimuli (DMA) are performed on both sides of the hands and feet. This in order to determine the changes in the sensibility of the skin, the sense of pain itself and sensitivity deficits with QST in the various hyperglycemic and euglycemic phases. The implementation of the QST is described in detail in page 15 of the study protocol.

The Examination-Clamp Test starts in the morning at 07:15 after a fasting period of 12 hours. Initially blood is drawn to determine plasma glucose, insulin, C-peptide, adrenaline, nor-

adrenaline, glucagon and cortisone levels. In order to construct the biobank and for the routine lab testing, blood tests are executed both at the beginning and end of the test. In addition, urine examination is also performed.

An intravenous glucose tolerance test (ivGTT) takes place, in which 1 ml / kg of glucose 30% is as bolus injected and the serum glucose is controlled over 1 hour, initially every 2 minutes and later in gradually prolonged intervals. Additionally blood tests for insulin, C-peptide and asprosin determination are executed. This test will be used to determine the pancreatic insulin reserve and to monitor asprosin kinetics during hyperglycemia.

Afterwards a glucose (20%) and an insulin solution (normal insulin 1.5 mU / kg body weight) is infused via an intravenous- cannula to the right elbow in order to set serum glucose manually at a target value of 90 mg / dl for a total duration of 1 hour. During this hour blood tests for the above mentioned hormones are performed. The same test is again performed with a glucose serum level of about 60 -70 mg / dl, corresponding to physiological fasting serum glucose values after prolonged fasting periods. This is done in order to be able to assess asprosin kinetics even at lower serum glucose levels. At the end of this last hour, blood tests for the measurement of serum insulin, C-peptide, asprosin, adrenaline, noradrenaline, glucagon and cortisone concentrations is performed. Including about two hours of follow-up after intravenous administration of Actrapid, the total duration of the examination is approximately 5 hours.

Indirect calorimetry (optional)

Measurement of O₂-uptake/CO₂-emission in a resting state and starving at the beginning, and again at the end of the clamp test for determination of substrate oxidation (glucose oxidation - GOX, lipid oxidation - LOX) in a resting state and with combined insulin and glucose infusion (Cosmed Quark RMR, Cosmed Deutschland GmbH, Fridolfing).

FibroScan® measurement (optional):

This is a pain-free, non.invasive clinical routine test of liver tissue using elastography (Echosens FibroScan®, Paris, Echosens™, Frankreich) for determination of liver stiffness, which can be used for steatosis follow up.

AGE measurement (mandatory):

Non.invasive fluorescence-based measurement of advanced glycation endproducts in the skin (DiagnOptics AGE Reader[®] SU, Diagnostics Technologies B.V., Groningen, Niederlande). No risks or contraindications are known for this method.

BIA measurement (not in patients with ICD or pacemaker):

Measurement of body components using bioelectrical impedance method (MEDI CAL Biacorus[®] RX 4000, MEDI CAL HealthCare GmbH, Karlsruhe). Sticky electrodes are attached to the patient's skin, resulting in minimal electric current. This method is widely used in clinical routine. For safety reasons, it is not used in patients with ICD or pacemaker. Other than that, no risks are known for this method.

Module 4: neuropathy module (optional)

- **NSS/NDS/SAS scores**

History of neuropathic symptoms using NSS and SAS scores, clinical detection by clinical-neurological testing using NDS score (monofilament, reflexes, cold-warm-discrimination, tuning fork).

- **Quantitative sensory testing and electrophysiological measurements**

Quantitative sensory testing (QST) is performed according to the protocol of the Deutscher Forschungsverbund Neuropathischer Schmerz (DFNS) (Rolke et al. 2006) for evaluation of subjective detection thresholds of sensory stimulation that allow to specifically test thick myelinated afferent fibers (low-threshold mechanic receptors for touch sensitivity and vibration), thin myelinated afferent fibers (cold detection, mechanical nociception), and non-myelinated afferent fibers (warm detection, heat-sensitive polymodal nociceptors). Testing includes thermal detection thresholds for cold and warm detection as well as paradox heat sensation, mechanical thresholds for touch sensitivity, vibration detection, and mechanical pain threshold (thresholds for bland pressure and needle-stings, dynamic allodynia, and summation of pain sensation in repeated stimuli).

Thermal detection, thermal pain thresholds

Measurement tool is a thermal sensory testing device (thermode). A standard test sequence is performed using a Thermal Sensory Analyzer (TSA by MEDOC, Israel). This

method is widely used in clinical research to detect the function of pain and thermal sensory fibers (C- and A-delta fibers) and has a CA-certification (TSA: CE 0473; MSA: CE 0413). Cold and warm detection thresholds are determined by this tool, as well as the thermal sensory limen (TSL) to detect paradox heat sensations (Fruhstorfer, Lindblom et al. 1976, Yarnitsky, Sprecher et al. 1995). Moreover, thermal pain thresholds can be tested using this thermode. For aforementioned testing, the thermode is applied to different areas (affected and non-affected) of the patient's skin. All thresholds are obtained by continuous up and down temperature stimuli (1°C/s) that are terminated when the study participant presses a stop-button. Cut-off temperatures are 0 und 50°C, with a baseline temperature at the beginning of all measurements of 32 °C. Contact area of the thermode is app. 13 cm². For thermal detection and pain thresholds, the mean value of a series of three measurements is used.

Determination of mechanical detection threshold

Measurement tool is a standardized set of modified von Frey hairs (0,25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 und 512 mN). The contact area of the von Frey hairs with the skin is of uniform size (<1 mm²) and shape (rounded tip to avoid sharp edges that would facilitate nociceptor activation). Nominated von Frey hairs (Optihair) are available by Marstock Co. (Marburg) and CE certified (CE, Class 1, 1999 granted). Mechanical thresholds are determined according to the method of limits using a series of calibrated von Frey hairs in five series of ascending and descending forces of application at affected and non-affected areas of the skin. Beginning with a force of application of 16 mN, von Frey hairs are applied in decreasing order until the patient does not perceive the stimulus anymore, which represents the mechanical detection threshold. Afterwards, von Frey hairs are applied in reverse order until the stimulus is detected by the study participant, which represents the second value of mechanical detection threshold. This process is repeated five times. In case the first force of application of 16 nM is not perceived, von Frey hairs with ascending forces of application are used until the stimulus is perceived, which then represents the first threshold value. Afterwards, von Frey hairs are applied with decreasing forces of application until the study participant does not perceive the stimulus (second value). These steps are repeated five times.

Determination of mechanical pain threshold

Mechanical pain threshold is measured using a set of seven metallic punctual mechanical stimulators (Pin Pricks) with standardized force of application (8, 16, 32, 64, 128, 256 und 512 mN), which have a cylindrical tip as contact area to the skin with no risk of skin injuries by appropriate use. Pin Pricks are used according to the method of limits, with the threshold being the geometric mean of five series of ascending and descending forces of application on affected and non-affected skin areas (Hampf, Bowsher et al. 1990, Ziegler, Piolot et al. 1999). The investigator uses Pin Pricks with different forces of application to detect the mechanical pain threshold. Only the needle itself and not the syringe barrel should be brought in to contact with the skin of the subject. The application and removal should be performed by a gentle movement leading to an application duration of 2 seconds. Beginning with the force of 8 mN, the stimuli are subsequently applied in increasing order of the intensity until the perception of the skin-contact is altered with an additional sharp sensation, at which point the applied force represents the first threshold value. Afterwards, Pin Pricks are used in reverse order (meaning descending forces of application) until the study participant perceives the stimulus without sharp sensation (second value). This procedure is repeated five times.

Determination of the mechanical pain sensitivity and dynamic mechanical allodynia for stroking stimuli/touch

Mechanical pain sensitivity is tested using the same set of Pin Pricks as used for mechanical pain threshold with the intensities of 8, 16, 32, 64, 128, 256 und 512 mN. An additional set of three light tactile stimulators including a cotton wisp exerting with a force of application of approximately 3 mN, a Q-tip (fixed to an elastic strip) exerting a force of application of approximately 100 mN, as well as a standardized brush (Somedic, Sweden) exerting a force of 200-400 mN is used for this testing (Rolke, Baron et al. 2006, Rolke, Magerl et al. 2006). These stimuli are applied in a fixed, balanced order for both affected and non-affected parts of the skin. This stimulus-response-function test is able to detect hyper- and hypoalgesia, as well as mechanical allodynia (Ziegler, Piolot et al. 1999). The Pin Pricks are applied as described above, whereas the tactile stimulators are applied with a single stroke (1-2 cm in length) over the skin. The study participant should give a pain rating for each stimulus on a verbal numerical rating scale (1-100). "0"

indicating “no pain” or “touch only”. The not-perceived stimuli should label ‘Ø’. “100” is indicating the most intense pain imaginable. Before the procedure, the finest and the most intense Pin Pricks as well as the cotton wisp, Q-tip and brush are demonstrated on the study participant on an exercise area.

Wind-up ratio

Measurement tool is a mechanical stimulator with standardized intensity (256 mN Pin Prick), which has a flat contact area (diameter 0.2 mm). Firstly, a single stimulus is applied with the 256 mN Pin Prick as described above, with the study participant rating the pain on a scale from 0-100 as described above. At intervals of 10 sec., trains of ten stimuli of the same force are applied repeatedly at a rate of 1/sec. within a small skin area of 1 cm². Directly after the subsequent train of stimuli, the study participant rates the pain again on the scale from 0-100. Out of the main pain rating of trains and the main pain rating of single stimuli, a quotient is calculated. This procedure is repeated five times. The wind-up ratio is calculated as the geometric mean of all five quotients (Magerl, Wilk et al. 1998). Before starting the actual procedure, it should be demonstrated on an exercise area.

Vibration detection threshold

Measurement tool is a tuning fork (64 Hz, 8/8 scale) as used in a clinical routine. To examine the vibration threshold, the tuning fork is placed on a bony prominence of a test- and control site (for example: on the feet on medial malleolus, on hands on processus styloideus ulnae, on head lateral on temples). Vibration threshold is determined by a series of three descending stimulus intensities (Fagiuz and Wahren 1981).

Detection of pressure pain threshold over skeletal muscles

Measurement tool is a bland mechanical stimulator with a contact area 1 cm², and an applied force of up to 20 kg/ 2000 kPa/ 200 N (or 10 kg/ 1000 kPa/ 100N when applied on the face), which has a scale for measurement of applied pressure (for example pressure algometer, Wagner Instruments, Greenwich, USA). This stimulator is applied to

an affected and an unaffected area of the skin over defined skeletal muscle areas (for example on the feet over the abductor hallucis muscle, or on the thenar of the hands, or over temporal muscle in the face). By applying constantly increasing pressure at a rate of 0.5 kg/sec. or 50 kPa/sec., pressure pain threshold of respectively tested will be defined.

Study participants who show signs of significant central nervous sensitization due to their QST profile will be asked to participate in further testing performed at the Neurophysiological institute (Director: Prof. Treede). There is an extra statement by the Ethics committee of the University of Mannheim for this additional testing, where it will be performed, so that they do not concern the University of Heidelberg.

Axonal excitability. One important parameter of planned testing is the excitability of axonal membranes of myelinated peripheral nerve fibers. For that, the method of “threshold tracking” will be performed, where the current will be analyzed via computer that is necessary for induction of an A-fiber summation potential with 40% of the largest possible amplitude (100%). Increase or decrease of this current due to simultaneous subliminal pre-depolarization shows an increase or an inhibition of excitability, respectively. The method of “threshold tracking” and the software needed (QTRAC; © Prof. Hugh Bostock, University London) have been established by our cooperation partners in Mannheim for several years (Fleckenstein et al, 2013). In addition to excitability, recovery phase of membrane threshold after an action potential of myelinated nerve fibers will be registered. For this, threshold of the current necessary for triggering a second action potential after a conditioning first action potential will be analyzed. Time gap between these two is variable between 10 and 400 msec. This recovery phase after an action potential is an indirect parameter for the membrane potential of myelinated nerve fibers. Main parameter is a change in relative refractory time of the recovery cycle. Changes in strength-duration time constant, rheobase current, refractoriness after 2 msec., superexcitability after 7 msec., as well as changes in threshold electrotonus.

Axonal excitability is measured by threshold tracking technique (Bostock et al, 1998) with the help of an multiple excitability measures (MEM) protocol (Kiernan et al, 2000), which is an automatic computer-based electric stimulation protocol (QTRAC Software, Institute of Neurology, London, UK). This protocol consists of a standardized sequence of electric

pulses, which are powerful enough to trigger A-fibers, but are still sub-threshold for thin C-fibers. Such evoked sensations are not painful, but are often felt as electrifying or tingling. Measurements are performed under supervision by a medical doctor and take about 10-15 minutes per site. Axonal excitability of motoric A-fibers is measured according to changes in summing potential of the abductor pollicis brevis muscle. Electrodes are placed over median nerve on the lower arm. Excitability measurements of sensory A-fibers are evoked by electrodes placed over either the median nerve, or the ulnar nerve on the wrist. Ring electrodes are used as lead electrodes, and will be placed on the base of the middle- or ring-finger.

For electric stimulation, a CE-certified (EN(IEC) 60601) constant current stimulation device (DS5 Digitimer) will be used. This device has a protection circuit, which only allows a defined maximum energy to be used for stimulation, independently of computer or user mistakes. For detection, a CE-certified (ICE 60601-1, 2000) AC-coupled EMG-amplifier (LP511, Grass Technologies) will be used. The power supply of this amplifier is medically regulated and uses DC voltage of ± 12 V.

Moreover, the same stimulation device will be used for determination of the sensitivity of skin nociceptors to depolarization. For this, sinus-wave-form electric stimuli with a duration of 0.5 sec. Will be applied to the skin by bipolar platinum-iridium stick electrodes (0,4 mm diameter, 1 cm length, 3 mm distance; Cephalon, Maastricht, Niederlande). Maximum current randomly varies between 0.2 and 0.8 mA, and will be repeated three times. After every stimulus, study participants will be asked to give a pain rating on a scale from 0-10, with 0 being „no pain“ and 10 being „the strongest imaginable pain“. Currents used are so weak (max. 0.8 mA) that no damage to the skin will occur. Expected maximum pain in healthy persons is around 3 out of 10. Study participants with diabetic polyneuropathy will usually feel less pain.

- **Heart rate variability:**

Determination of heart rate variability as surrogate parameter for autonomic neuropathy (SUESS SUEmpathy[®] 100, SUESS Medizin-Technik GmbH, Aue). For this, a normal 3-lead ECG will be used, so that this method is completely non-invasive.

- **Measurement of nerve conduction velocity of sural nerve, peroneal nerve, and tibial nerve:**

By use of sticky electrodes, minimal electric current will be applied, which is only felt as tingling by the study participants (Viasys Healthcare VikingQuest[®], Viasys Healthcare GmbH, Höchberg). This examination will not be performed in persons with ICD or implanted pacemaker devices. Otherwise it is free of risks and frequently used in clinical routine.

Module 5: Vascular module (mandatory)

- **Physical examination**

Palpation of the following pulses on both legs: Femoral artery, popliteal artery, dorsal pedic artery, posterior tibial artery. Non-invasive measurement of blood pressure by Riva-Rocci method, and pulse by palpation.

- **Intima-Media-thickness (IMT) of carotid artery**

Determination of IMT by use of high-resolution ultrasound technique (Samsung SonoACE[®] X8, Samsung Medison Co. Ltd.) as important surrogate parameter for vascular complications. For measurement, internationally accepted protocols are used.

- **Doppler ultrasound of carotid artery**

By use of ultrasound (Samsung SonoACE[®] X8, Samsung Medison Co. Ltd.), blood flow velocity of carotid artery will be examined in order to detect plaque or stenoses. Stenoses will be classified according to NASCET-criteria.

- **Abdominal ultrasound**

Abdominal ultrasound (Samsung SonoACE[®] X8, Samsung Medison Co. Ltd.) by use of a 3.5 MHz probe will be performed with focus on the following parameters: Size and morphology of the kidneys, perfusion of the kidneys, diameter of the infrarenal abdominal aorta, and liver parenchyma (NAFLD).

- **12-lead ECG**

Key parameters are cardiac rhythm, indication for coronary artery disease or former myocardial infarction (Mortara Instrument ELI250, Mortara Instrument GmbH, Essen).

- **Ankle-brachial-index (ABI-Messung):**

Non-invasive blood pressure measurement on arms and legs by use of Riva-Rocci method (BOSO ABI-System 100, Bosch + Sohn GmbH, Jungingen). This examination will not be performed on study participants with lymphedemas or after lymphadenectomy. Otherwise it is free of risks without further known contraindications.

- **Fundus photography**

Capturing retinal pathologies by use of central 1-field-photography (NIDEK AFC-230[®], NIDEK CO LTD, Padova, Italien; Canon EOS 5D Mark II, Canon Deutschland GmbH, Krefeld). For this method, no mydriasis is necessary, so that no eye drops will have to be used, and no direct contact to the eye will be necessary. Therefore, this method is free of risks, non-invasive, and widely used in clinical routine.

Module 6: Pulmonary examination (mandatory in case of no contraindications)

- Clinical auscultation of the lung with a stethoscope.

- **Lung function testing**

Lung function testing will be performed by bodyplethysmography (Ganshorn PowerCube[®] Body+ und Diffusion+, Ganshorn, Niederlauer). This is a non-invasive clinical routine method. It is not performed in case instructions are not understood by the study participant, or if the study participant exceeds a body weight of 160 kg since this is the maximum weight for which the cabin is approved.

- **Pulsoxymetry**

Non-invasive measurement of oxygen saturation by use of a finger clip (Pulox[®] PO-250, Novodion GmbH, Köln).

6.2 Duration of the study

It is a cross-sectional study. The duration of the study is so long that all participants are recruited and examined.

6.3 Selection of study participants

Inclusion criteria for participants without hypoglycemia unawareness

- Age between ≥ 18 and ≤ 75 years
- BMI between 20 und 35 kg/m²
- Persons with manifest diabetes mellitus type 1 and diagnosis according to DDG guidelines 2011 oGTT, HbA1c $\geq 6.5\%$ in the absence of adulteration of the HbA1c, over 200 mg / dl in the 2 hour value of the oGTT, fasting glucose > 126 mg/dl, spontaneous glucose > 200 mg/dl at least twice).

Inclusion criteria for participants without hypoglycemia unawareness

- Persons who are not aware of hypoglycemia symptoms at glucose levels above 50 mg / dl
- Age between ≥ 18 and ≤ 75 years
- BMI between 20 und 35 kg/m²
- Persons with manifest diabetes mellitus type 1 and diagnosis according to DDG guidelines 2011 oGTT, HbA1c $\geq 6.5\%$ in the absence of adulteration of the HbA1c, over 200 mg / dl in the 2 hour value of the oGTT, fasting glucose > 126 mg/dl, spontaneous glucose > 200 mg/dl at least twice).

General exclusion criteria

- Secondary types of diabetes (ADA-criteria type 3 B-H)
- Current pregnancy
- Acute infections / fever
- Immune-suppressant therapy
- Severe psychiatric diseases requiring treatment (for example personality disorders, schizophrenia, depression)
- Known alcohol or drug dependency
- Severe heart-, kidney-, or liver-insufficiency:
 - NYHA stadium IV
 - Non-diabetic liver disease (for example PBC, PSC, Wilson's disease, hemochromatosis, autoimmune hepatitis)

- severe peripheral artery disease (stadium IV)
- non-diabetic glomerulopathy
- Cancer or other malignant diseases within the last 5 years
- Infectious diseases like hepatitis B, C, E, or HIV
- Other severe autoimmune diseases
- Current participation in an interventional study
- Anemia or disorders of bone marrow
- **Exclusion criteria for clamp study:**
 - Past history of deep vein thrombosis or pulmonary embolism
 - Routine laboratory test results $\leq 80\%$ below lower reference value: Ferritin, iron, leucocytes, haemoglobin, hematocrit, RBC, platelets, blood alcohol levels.
- **Exclusion criteria for bioimpedance measurement:**
 - Pacemaker / ICD
- **Exclusion criteria for lung function testing:**
 - Ignoring or non-understanding of the instructions

6.4 Course of the study

One day is scheduled for basic testing. Study participants are scheduled to arrive between 11 and 12 am in a fasting state. The basic examinations take three to four hours, dependent on the total number of patients in the outpatient clinic since some devices have to be shared with clinical routine. QST takes 90 minutes and can be performed on the same day as basic testing. On a second day a hyperinsulinemic clamp examination is performed, which will be scheduled separately, within four weeks of basic testing. The clamp lasts about 5 hours including the follow-up time. Initially blood is drawn in order to measure of plasma glucose, Asprosin, insulin, C-peptide, adrenaline, norepinephrine, glucagon and cortisone concentrations, and twice for the biobank (at the beginning and end of the test) and for determining the values of the routine lab testing will be performed. In addition, a urine examination takes place. All tests will be performed according to SOPs by trained personnel. All participants are informed of the driving prohibition for the rest of the day after enduring the Clamp test.

6.5 Potential complications and risks

This study will yield new data on the new hormone asprosin und the Pathomechanism of the hypoglycaemia unawareness. Under consideration of the limitations, we strive to identify different subgroups of type 1 diabetics with or without hypoglycaemia unawareness, which would

be separated on the basis of combinations of specific biomarkers and genetic characteristics and therefore bear either a high or a low risk for the development of diabetes-associated complications. This could have an impact on and direct consequences for specific differential therapies and specific preventive strategies. Expected medical complications are rather rare and only mild. In case of venous blood draws and placement of iv-cannulas, there is a risk for hematomas, infections, and very rarely phlebitis or thrombophlebitis. Even rarer is a temporary or even permanent damage of small nerve fibers in the skin.

In the clamp study, total blood volume will not exceed 270 ml including blood for the biobank. . This volume is the equivalent of half a blood donation, which is usually well tolerated, so that the total amount of blood drawn should not real to complications.

All stimuli applied during the QST are close to the pain threshold and therefore tolerable for the study participants. Moreover, study participants have the possibility to abort the test at any time point. Painful stimuli can lead to a slight temporary local flush of the skin.

During optional clamp testing, hypoglycemia can occur during or shortly afterwards. Moreover, hypokalemia can occur due to simultaneous infusion of insulin and glucose. Both complications can be treated immediately and effectively by supplementation of glucose (which is always running anyway) and oral supplementation of potassium, respectively.

6.6 Stopping criterion

The only reason is for a study participant to withdraw from study participation. In this cross-sectional study is that not to be expected.

6.7 Concomitant therapy

The study does not influence the study participants' regular medication of other medical treatment in any way.

7. Statistical analysis and sample size calculation

The number of 10 subjects suffices for this pilot project. The aim of the study is to assess the asprosin effect during the hyperinsulinemic clamp test as well as its correlation to the rest of

Asprosin dynamics relating to serum Glucose levels under controlled alteration from Version 1.1 Stand 31.05.17

known regulatory hormones. Because of the low number of participants, only non-parametric evaluations, as well as descriptive and graphical statements, are used. Depending on the parameter, relating or non-relating non-parametric tests are performed. It is a pilot study in which no large statistical calculations are possible or, currently necessary. No biometrics will be involved in the evaluation.

The effect variables determined in this study, in particular asprosin kinetics under different glucose related metabolism conditions, can be further used for possible power analysis of larger future studies.

8. Data collection and data management

All collected data from the study will be transferred into a case report form (CRF), which contains an unequivocal participation number. Moreover, all samples will be labeled with a code number, so that all analyzes can be performed by use of a pseudonym. Questionnaires containing personal data will be stored separately. The code can only be associated with the study participant by a small group of direct employees of the Study Center for Diabetes Research of the University of Heidelberg. Decoding only takes place in case of withdrawal from study participation in order to erase all data, or in case of severe medical findings about which the study participant has to be informed. In statistical analyses and publications, data will be completely anonymous. Anonymization will be performed during creation of excel sheets of SPSS tables, which do not contain a pseudonym anymore. All data and results from the study are subject to professional secrecy. Results in the context of medical publications will only be presented as statistical entities. Study files will be stored in a lockable room, which can only be accessed by employees of the Study Center for Diabetes Research of the University of Heidelberg. Clinical data will be stored for 15 years.

Moreover, a biobank will be created. Samples will be stored at -80°C in designated freezers up to 5 years, labeled by use of pseudonyms. The pseudonym is the same used for clinical data. From material stored in the biobank, different analyses associated with glucose metabolism are planned, especially concerning the regulation of different pathways, production and concentrations of reactive dicarbonyls, as well as the analysis of reactive oxygen species. Specific scientific questions, however, cannot be appointed at this point. Therefore, we reserve the right to perform other analyses, for example in case alternative substances or metabolic pathways are detected during the running period of the study.

Electronic documentation of the data will be performed using a Microsoft Access® database (Microsoft Deutschland GmbH, Unterschleißheim), which has been created locally in Heidelberg. Database hosting including backups will take place at the office of the Clinical Study Center for Diabetes Research of the University of Heidelberg, Department Medicine I. Data will be stored on an external hard drive, with backup on a second external hard drive. For encryption of the data, a pseudonym will be used. Data will only be accessed and used by employees of the Clinical Study Center for Diabetes Research of the University of Heidelberg. The external hard drives will be stored secured in the office of the Clinical Study Center for Diabetes Research of the University of Heidelberg, to which only a small group of employees in this study center have access. Data will be stored electronically as described, a CRF on paper is only planned for emergencies (power failures) and for parts of the questionnaires that have to be filled in by the study participants themselves.

9. Legal regulations and insurance

All study examinations will be performed according to the medical professional act and in accordance to the Declaration of Helsinki (2013).

All study participants will be informed about the aims of the study, the conduction of the diagnostic tests and the blood draw, as well as the risks associated with them before enrollment by the study doctor. The patient is enrolled in the study after obtaining written informed consent. Study participation is completely voluntary, and study participants can withdraw from study participation any time without specific reasons and without negative consequences concerning future medical care. In case of withdrawal, study participants can decide whether their biomaterial will be destroyed or can be used anonymously for ongoing medical research. The names of the study participants, as well as all other confidential data are subject to medical confidentiality and regulations of the German Federal Data Protection Act. Personal data will only be passed on by use of a pseudonym, and only in case specific analyses using biomaterial cannot be performed at the University of Heidelberg due to technical or organizational reasons. In that case, material can be sent to other research facilities. This is specifically explained in the information form for study participants, which will be handed to every person interested in participation before enrollment. Third parties will not get access to original data and files.

Compensation insurance for accidents en route has not been contracted.

Heidelberg, 31/05/2017



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Research
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