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"Recommendations for measurement of Ghrelin"

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Zusammenfassung

Hintergrund und Ziel:

Ghrelin ist ein Hormon das bei Hunger und Sättigung eine große Rolle spielt. Seit der Entdeckung 1999 wurden viele Studien durchgeführt, um die Funktion und potentiellen positiven Wirkungen des Hormons zu untersuchen. In den meisten Studien wurde für die Ghrelinmessung ELISA oder RIA verwendet. Für die vorliegende Arbeit sollte der Einfluss von Mental Imaging auf die Ghrelinwerteuntersucht werden. Das Hormon wurde in Blut und Speichel gemessen, aber die Resultate konnten nicht für eine wissenschaftliche Aussage verwendet werden. Deshalb konzentriert sich diese Arbeit auf die verschiedenen Analysemethoden und Probenvorbereitungen von Ghrelin.

Methode:

Die Literatursuche wurde im Internet in den folgenden Datenbanken durchgeführt: PubMed, Science Direct und Embase. Jede Studie musste definierte Einschluss- und Ausschlusskriterien erfüllen, um in der Arbeit verwendet zu werden.

Resultate:

Insgesamt 31 Studien wurden für diese Arbeit verglichen. Gesunde und adipöse Probanden wurden untersucht. Die Ergebnisse der Studien waren zum Teil sehr unterschiedlich. Konzentrationen, die mit RIA gemessen wurden, waren zwischen 478,49pg/ml und 1129,4pg/ml bei gesunden und 543pg/ml und 952pg/ml bei adipösen Teilnehmern. Bei der ELISA-Methode waren die Werte bei gesunden zwischen 229,1 und 48840pg/ml bei adipösen Probanden. Besonders bei den Werten im Speichel gab es eine große Spannweite: die Werte lagen zwischen 87 und 67000pg/ml. Viele Faktoren können einen Einfluss auf die gemessenen Ghrelinwerte haben: das Älterwerden, Ernährung, Probenbereitung, Analysemethoden, die Verwendung von Proteaseinhibitoren, Temperatur, pH-Wert und andere Einflussfaktoren können die Messungen beeinflussen.

Fazit:

Ghrelin könnte eine Therapiemöglichkeit für diverse Krankheiten darstellen, deswegen ist es sehr wichtig die Funktion des Hormons zu verstehen und eine standardisierte Analysemethode zu finden. Es sollte mehr Studien mit einer großen Probandenzahl geben um eine gute Methode zu finden die reproduzierbare Ergebnisse liefert. Nur dann ist es möglich die Funktion und möglichen therapeutischen Nutzen richtig zu erforschen.

Keywords: ghrelin, ELISA, RIA, adipös, gesund, Blut, Plasma, Speichel

Abstract

Background and objective:

Ghrelin is a peptide hormone that plays an important role in hunger and satiety. Since it was discovered in 1999 a lot of studies were performed to investigate the function and possible benefits of ghrelin. In most studies ELISA or RIA was used to measure the hormone. In this thesis we wanted to investigate the influence of mental imaging on ghrelin value in the body. Ghrelin was measured in saliva and blood, but our results could not be used for a scientific statement. That is why this thesis is concentrating on the possible influences of different analysis methods and sample preparation of ghrelin to find the best way to measure ghrelin.

Method: Literature search was made on the internet in following databases: science direct, pubmed and embase. Every publication had to meet special inclusion and exclusion criteria to be part in this work.

Results:

Overall 31 publications were included for comparison of ghrelin values. Healthy and obese subjects were investigated. The results were very different, especially with ghrelin values measured with ELISA. Concentrations measured with RIA were 478.49pg/ml to 1129.4pg/ml in healthy subjects and 543pg/ml and 952pg/ml in obese subjects. For analysis with ELISA results were 229.1 to 48840pg/ml for healthy participants and 478.5 pg/ml and 1129.4pg/ml for obese patients. Especially concentrations measured with ELISA were very wide spread. There were only 4 studies that included ghrelin concentrations in saliva. The values were between 87 pg/ml and of 67000 pg/ml. Many different factors can be the reason for the different outcomes of ghrelin concentrations. Factors such as senescence, food intake, sample preparation, analysis method, the use of protease inhibitors, temperature, pH-value and other elements could influence ghrelin values.

Conclusion:

Ghrelin could be a promising therapeutic option for different diseases, hence it is very important to get a better understanding of the hormone and a standardized method of analysis. There should be more research to find a good method that delivers reproducible results. Only then it is possible to investigate the function and possible therapeutic value of ghrelin.

Keywords: ghrelin, ELISA, RIA, obese, healthy, blood, plasma, saliva

List of abbreviations

AC	Acylated Ghrelin
AgRP	Agouti related Protein
ARC	Arcuate Nucleus
BMI	Body Mass Index
CCK	Cholecystokinin
CHF	Chronic Heart Failure
EIA	Enzyme Immunoassay
ELISA	Enzyme linked immunosorbent assay
GH	Growth Hormone
GHS-R1a	Growth Hormone Secretague Receptor 1a
GOAT	Ghrelin O-Acyltransferase
HCl	Hydrogen Chloride
HCN	Hydrogen Cyanide
HC	High Carbohydrate
HF	High Fat
HP	High Protein
LH	Lateral Hypothalamus
MBOAT4	Membrane Bound O-Acyltransferase Domain Containing 4
mRNA	messenger Ribonucleotide Acid
MMC	Migrating Motor Complex
NA	Not Available
NPY	Neuropeptide Y
da	Eingefügt
pH	potentia Hydrogenii

PIs	Protease inhibitors
POMC	Propriomalonocortin
PVN	Para Ventricular Nucleus
PWS	Prader Willi Syndrome
PYY	Peptide YY
RIA	Radioimmunosorbent Assay
T2DM	Type 2 Diabetes Mellitus
UAC	Unacylated Ghrelin
GI	Gastrointestinal
WHO	World Health Organization

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1. INTRODUCTION AND QUESTION

INTRODUCTION AND QUESTION

In 1999 ghrelin, a growth-hormone-releasing peptid, was discovered by Kojima and Kangawa. It was discovered as the endogenous ligand for growth hormone secretagogue receptor (GHS-R) in the stomach. At first, it was described only as a inducer of growth hormone (GH) secretion (Kojima et al.,1999) but shortly thereafter the connection between food intake, adiposity and ghrelin was made (Nakazato et al., 2001). Since then, plenty of studies were made, to get more information about the relationship between ghrelin and hunger, bodyweight, appetite, glucose metabolism, adiposity or eating disorders.

In 2016, my colleague Julia Bichl and me wanted to test the impact of food visualisation on eating behavior. We wanted to examine if it is possible to quantify the outcome of a previous publication by Missbach et al. (2014) physiological. Here, participants who imagined consuming a certain food resulted in decreased food intake in comparison to participants who imagined putting coins into a machine.

Schüssler and colleagues investigated a significant increase of ghrelin after a visual presentation of food (2012).

We wanted to investigate if one can determine this theory by measuring ghrelin in human saliva and plasma. First, we intended to perform a pre-study to get an idea of the average amount of ghrelin levels in human plasma and saliva. This was important because previous publications about showed a big variation between the measured hormone-values. A doctor was brought in to take blood samples from volunteers.

We used an EIA-kit from Sigma-Aldrich (now Merck) and followed the instruction steps, including all recommendations. Unfortunately, the difference

between our outcome values was so substantial (also of the same sample, we measured in duplicate) that we could not use the results for a scientific statement.

The ghrelin values of the standard curve were measured in triplicate as shown in figure 1. There were some problems with the standard cure, especially with the 1000pg/ml concentration.

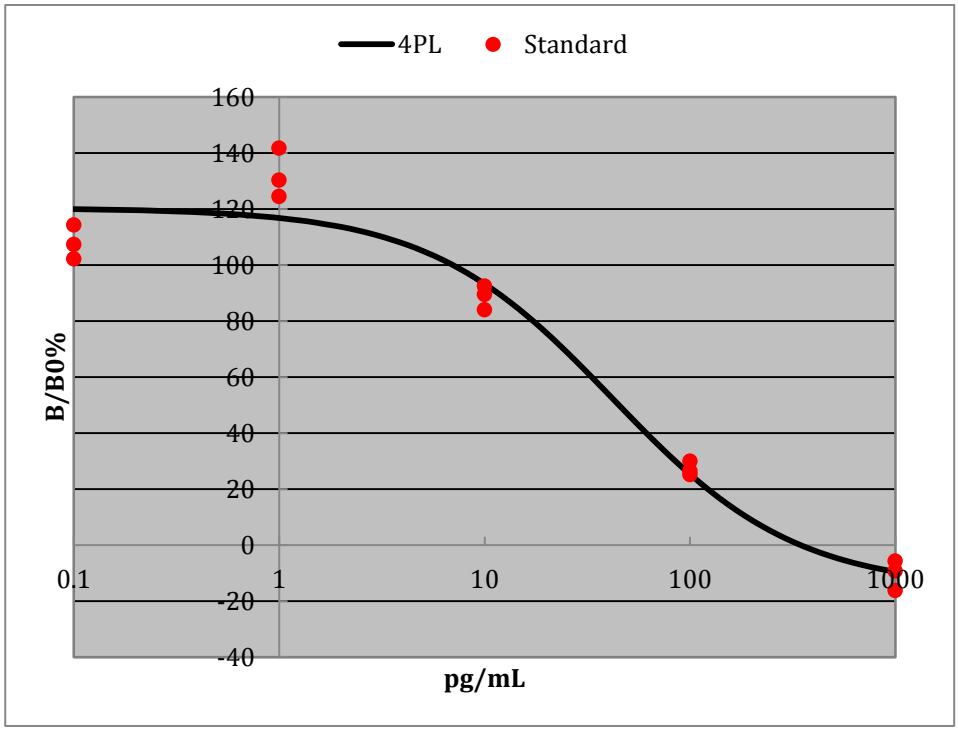

Figure 1: standard curve for ghre measurement

Our values reached from 18pg/ml to 504pg/ml. Some values could not be evaluated because they were above the standard curve. Samples were collected in different blood collection tubes, some with heparin or EDTA and some without any protease inhibitors. Some saliva samples were measured in triplicate.

We could not figure out the problem during our pre-study, that is why this systematic review concentrates on the different methods of ghrelin measurement.

The best known method for ghrelin-measurement is the analysis with ELISA/EIA or RIA. Some studies also used LC-MS or Luminex. This systematic review will focus on analysis with RIA and ELISA.

An important step of hormone analysis is the preparation of different samples. There are different ways to prepare ghrelin samples. Some ELISA- or RIA-kit producing companies recommend the use of protease inhibitors (PIs), such as aprotinin, AEBSF, PHMB or PMSF. Often, the use of HCl or another acid is recommended to create a lower pH value or a specific storage temperature of the plasma and saliva sample, so this work will also look into the right treatment of blood and saliva samples. Ghrelin is unstable at room temperature, that makes a fast and correct way of sample preparation inevitable.

The purpose of this systematic review is to figure out which is the best and most promising way, to measure ghrelin in human plasma.

2. LITERATURE SURVEY

2.1 Ghrelin

In 1999 the peptide hormone ghrelin was discovered by Kojima et al.. It was identified as the endogenous ligand of GHSR-1a (growth hormone secretagogue receptor 1a). This G protein-coupled receptor stimulates the release of growth hormone from the pituitary through growth hormone secretagogoues (GHS) which are small synthetic molecules (Kojima et al., 2001).

In comparison with other gastrointestinal peptides (like cholecystokinin or insulin) and adipocytokines (like leptin) ghrelin is the only known circulating hormone with orexigenic effects: it stimulates growth hormone (GH) secretion by pituitary cells, appetite, energy expenditure, weight gain and food intake. It also plays a major role in inflammation, circulation or digestion (Peeters, 2005).

2.1.1 Structure and signaling cascades of ghrelin

Ghrelin is a peptide hormone with 28 amino acids. Its serine-3 is n-octanoylated, this molecule constellation is essential for the hormones binding on GHSR1a and it plays an important role for ghrelins function. This modification is the first of its kind in mammals and is indispensable for ghrelins activity. Human ghrelin gene consists of five exons and is localized on chromosome 3p25-26 (Sato et al., 2011).

The hormone is mainly secreted in the stomach by A/X-cells (Van der Lely et al., 2004) and then enters the blood circulation to reach the GHS-R1a receptor in hypothalamus. Other ghrelin expressing organs are duodenum, ileum, jejunum, colon, pancreas, as well as kidney, hypothalamic arcuate nucleus, and placenta. The binding on GHS-R1a receptor leads to signaling cascades,

causing the release of neuropeptides and neurotransmitters like neuropeptide Y(NPY), proopiomelanocortin (POMC), agouti related protein (AgRP) or orexin, which leads to appetite sensation (Nakazato et al., 2001).

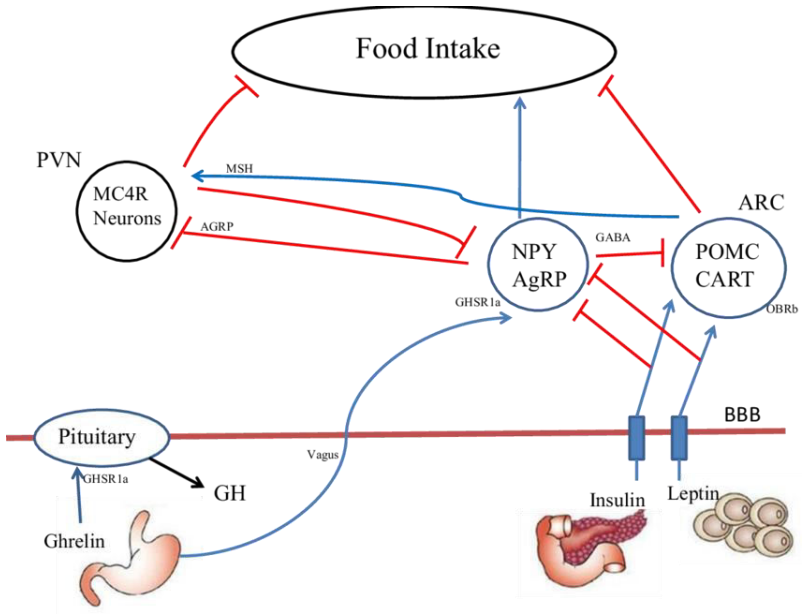

Figure 2: simplified visualization of the regulation of feeding behavior (Frago & Chowen, 2015)

Ghrelin derives from proghrelin, which is enzymatically cleaved between Arg28/Ala29 to result in unacylated and acylated ghrelin (Bang et al., 2007). Through the enzyme O-acyltransferase (GOAT) an octanoyl modification is then added to Ser^3 (Castaneda et al., 2010).

GOAT is coated by membrane bound O-acyltransferase domain containing 4 (MBOAT4) gene. GOAT is widespread in the human body and found in several human tissues (stomach, breast, colon, duodenum, jejunum, ileum, fat, kidney, liver, lung, muscle, pancreas, prostate…). The widespread distribution of ghrelin expression corresponds with the widespread expression of GOAT. The expression of GOAT is high in gut and stomach, which are as well the most important ghrelin-secreting tissues, and in the pituitary, where ghrelin shows paracrine and autocrine effects (Kojima and Kanagawa, 2005).

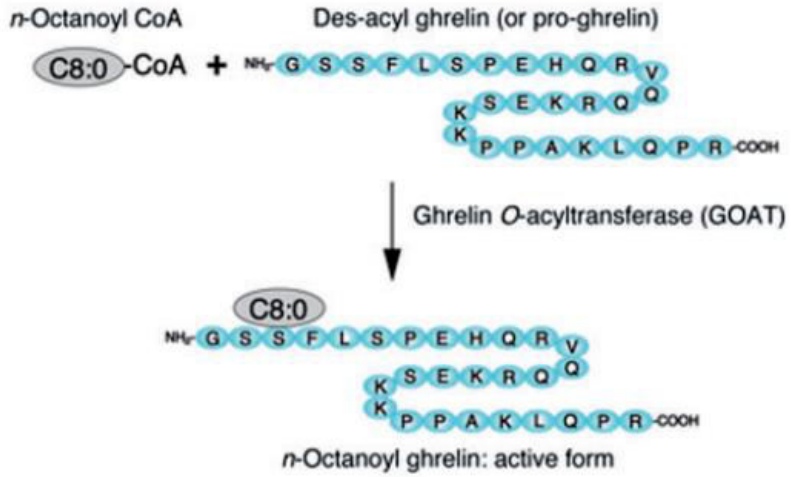

Figure 3: Modification of Des-acyl ghrelin by GOAT with an acyl acid to get active ghrelin (Kojima et al., 2016)

Obestatin is then cleaved from C-ghrelin, the carboxyl terminus C-ghrelin (acylated ghrelin) (Castaneda et al., 2010). Obestatin, a 23-amino acid hormone, also plays a role in hunger and satiety. It was believed to suppress hunger but its functions stay controversial. It is released in the stomach and found in many tissues and serums, especially in the gastrointestinal tract but also in the mammary gland, breastk milk, spleen and plasma (Lacquaniti et al., 2011).
Both, ghrelin and obestatin, are encoded by the GHRL gene (Zhang et al., 2005).

2.1.2 Different forms of ghrelin

Ghrelin appears in different forms in blood circulation: non acylated (des-acyl ghrelin), octanoylated, hexanoylated, decanoylated, and decenoylated (Kojima and Kangawa, 2005); but only the first two are existing in relevant concentrations: the two major forms are des-acyl ghrelin and n-octanoyl-modified ghrelin. These ghrelin forms with modifications on Ser3 were all found in the human stomach. Des-acyl ghrelin exists in blood circulation in higher concentrations, but octanoylated ghrelin is the active form (Staes 2010; Sato

2005).

Des-acyl ghrelin constitutes about 80% of circulating ghrelin. First it was thought to be biological unactive, since it was not able to bind to GHSR-1a. But it is able to bind to another receptor, a ghrelin receptor subtype, distinct from GHSR-1a which is found in cardiomyocytes and endothelial cells.

It has similar binding affinities to both forms: acylated and des-acylated ghrelin (Stasi and Milani, 2016).

Studies have shown that the elimination of 14 C-terminal amino acids of ghrelin does not change the impact of its bioactivity in vitro a lot, but a change in the octanoyl modification to other PTMs influences its receptor binding (Bednarek et al., 2000). The peptide deriving from N-terminal of the ghrelin molecule, which consists of four residues has the ability to interact with the receptor of ghrelin, especially the ester bond between the octanoyl part and Ser 3 as well as Phe, the fourth residue, are important for receptor activation (Kojima and Kangawa 2005).

The degradation of ghrelin are different enzymatic processes such as des-octanoylation and proteolysis. The half-life of ghrelin is estimated to be between 5 and 15 minutes. N-octanoyl-modified ghrelin is acylated on its third serine residue through the membrane-bound enzyme GOAT (Horvath et al., 2003; De Vriese et al., 2004).

Binding and activation of ghrelins receptor (GHSR-1a) is only possible through acylation. GHSR-1a receptors are expressed primarily in the pituitary gland and hypothalamus and stimulate the effects of ghrelin on GH-release and food intake (Khatib et al., 2014). GHSR-1a mRNA expression is also found in peripheral organs like lung, heart, uterus, liver, gonads, kidney, intestine, immune cell, pancreas, stomach an adipose tissues in a smaller amount (Papotti et al., 2000).

With the considerations of the points above, I want to discuss the current analytical methods, sample collections, detection, separations, measurement, storage conditions and so on for ghrelin to make a suggestion for the best handling of ghrelin in human plasma and saliva.

The many different functions of ghrelin makes it and its pathways interesting for potential therapeutic use. Especially for disorders like obesity, eating disorders or cachexia, the usage of ghrelin seems promising. Ghrelin has also shown beneficial effects on cardiac performance in patients with chronic heart failure (CHF). It could also help patients with CHF by the induction of positive energy balance and reduce negative consequences of cardiac cachexia (Nagaya et al., 2003).

2.1.1 Function of Ghrelin

There are numerous studies about ghrelins effect on energy metabolism. Some human studies have shown increased hunger and food intake after the administration of ghrelin in patients with anorexia nervosa (Hotta et al., 2009), patients with peritoneal dialysis (Wynne et al., 2005), patients with cancer (Neary et al., 2004). One study showed increased hunger sensation in healthy, lean, obese and malnourished test persons (Wren et al., 2001). Fasting ghrelin seems related to hunger-modulated activity in specific brain regions. The activation of hypothalamus, prefrontal cortex and amygdala by ghrelin suggest there is more to the enhanced food intake than only hunger and satiety, because these three brain regions are also reward centers.

As one can see in figure 3, ghrelin has many more physiological effects in the human body. It lowers insulin secretion and elevates insulin sensitivity. It also plays a role in glucose metabolism, gastric emptying, cardiac output, thermogenesis and other physiological pathways, a lot of them being a part of hunger and satiety.

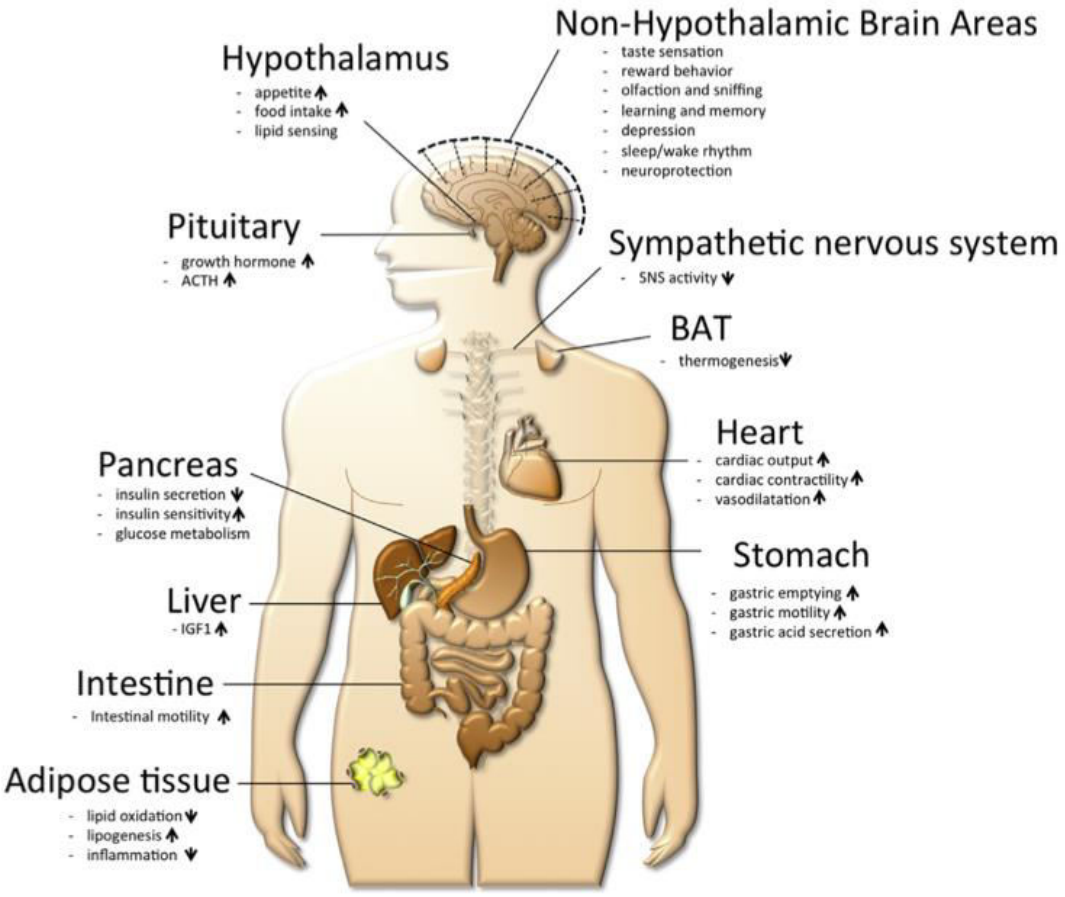

Figure 4: Ghrelins physiological effects (Müller et al., 2015)

Ghrelin and glucose metabolism: Ghrelin and the ghrelin receptor are expressed in various tissues like the brain, kidney, lung, heart, peripheral tissues, intestine, ovaries or pancreatic islets. Some human studies showed amount of glucose in plasma and decreased amount of insulin after ghrelin administration (Broglio et al., 2001; Broglio et al., 2003). The alpha-cells of the pancreatic islet express GHSRs and probably are the reason for the direct stimulation of glucagon secretion by ghrelin (Chuang et al., 2011). There seems to be a reciprocal connection between ghrelin and insulin levels in plasma, as they go through reciprocal changes during the day or hyperinsulinemic clamps (Cummings et al., 2001).

Ghrelin and GI-motility: ghrelin is very similar to motilin, a gut hormone that regulates migrating motor complex (MMC). MMC appear in the interdigestive state and are waves of electrochemical activity (De Smet et al., 2009). Because of the homology between ghrelin and motilin it was hypothesized that ghrelin has similar effects to motilin on motility. Some studies have shown faster gastric emptying and the induction of MMC in a fasted state after the intravenous administration of ghrelin (De Smet et al., 2009; Levin et al., 2006). Therefore ghrelin administration could help individuals with hypomotility disorders.

Ghrelin and food intake: studies on rodents have shown an increase in food intake and adiposity after ghrelin administration (Tschop et al., 2001). Ghrelin has shown increased activity in neuron expression like Agrp and Npy while inhibiting others. The administration of ghrelin to mice that lack Agrp and Npy failed to show increased food intake. Agrp and Npy are found in the ARC, a hypothalamic center that regulates food intake and satiety (Chen et al., 2004). Central ghrelin administration shows increased stimulation of enzymes that play a role in fatty acid storage while the fat oxidation is decreased. The effects of ghrelin on adiposity are a result of the mediation of signaling mechanisms of the melanocortinergic system, food intake and the regulation of lipogenesis (Theander-Carollo et al., 2006).

Other studies have also shown a link between ghrelin and memory, learning, stress, mood, depression, anxiety, aging or the sleep and wake rhythm (Sominsky and Spencer, 2014; Lutter et al., 2008; Szentirmai et al., 2007).

2.1.2 Measurement of Ghrelin

There are different methods of measurement of ghrelin: the most common are Radio Immunoassay (RIA) and Enzyme-linked immunosorbent assay (ELISA), but also Luminex and different high performance liquid chromatography (HPLC) are used.

Radio immunoassay (RIA)

RIA was developed by S.A. Berson and R.S. Yalow in the late 1950s to detect insulin in human serum. Yalow also received the nobel prize for their invention after Bersons death (Oldelberg, 1978).

An antigen-containing sample of interest, a complementary antibody and a radiolabeled version of the antigen is required to perform RIA.

How does it work:

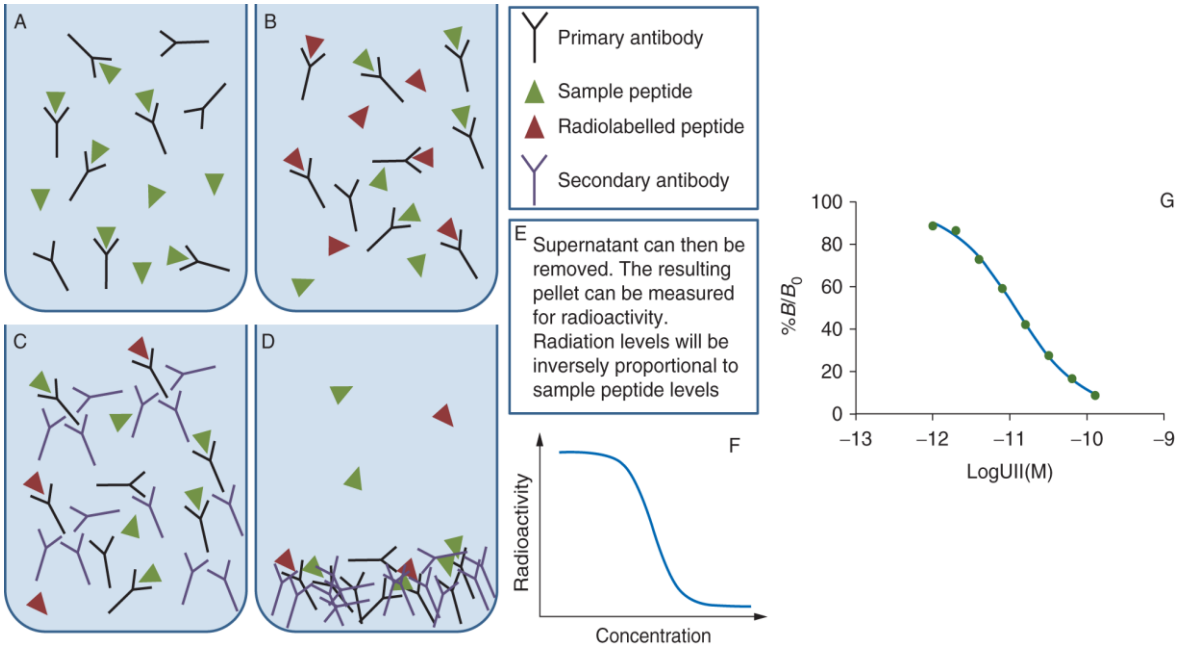

Figure 5: The principle of RIA (Grange et al., 2014)

The antibody and the sample antigen are put together for a specific incubation time to allow the antigen to bind with the antibody. Then the radiolabeled antigen is added. A competition between the radiolabeled antigen and the sample antigen starts, both want to bind with the antibody. The less sample antigen is present, the more radiolabeled antigen is able to bind to the antibody. Then a second antibody is attached that binds the primary antibody to allow the separation of the primary antibody. The centrifuging of the mixture is possible due to different density of the antigen-antibody complex and the antigen-free solution. A pellet remains containing the complex of sample antigen and radiolabeled antigen. Measurement of radioactivity of the pellet allows to determine the amount of radiolabeled antigen and therefore the concentration of sample antigen.

A disadvantage of RIA is the use of radioactive materials and therefore a relatively short shelf life. In addition it is considered less safe due to the radioactive materials (Grange et al., 2014).

Enzyme-linked immunosorbent assay (ELISA)

ELISA and as well enzyme immunoassay (EIA) are important analytical tools for detection and quantification for and in medicine, biomedicine or quality control, for example for the detection of hormones, peptides or proteins in blood. EIA and ELISA are similar analysis methods and both derive from RIA. EIA/ELISA work with an enzyme instead of a radioisotope which is used in RIA. Therefore, EIA/ELISA appear to be a safer analytical method because there is no need to use radioactive material anymore. EIA/ELISA are immunoassays methods, a biochemical analysis method which uses antibodies or antigens.

<u>HOW IT WORKS:</u>

With EIA/ELISA it is possible to detect a really small amount of antigens such as hormones, proteins, peptides or antibodies. The concept is the binding of an antigen to its specific antibody, which results in a color change. Prior the antibody is marked with an enzyme, most commonly used enzymes are glucose

oxidase, horseradish peroxidase and alkaline phosphatase. There are different kinds of ELISA such as competetive or sandwich ELISA but the principle is the same for all of them: It starts with the immobilization of antigens to a microplate well surface. Plate blocking is following: a protein or another molecule is added into the wells to cover the unsaturated surface-binding sites. A incubation phase follows with antigens that are specific for the used antibodies that bind to the antigens. The generated signal can be measured via a tag (direct or secondary) on the specific antibody.

The enzymatic reaction leads to a color change or fluoresence, indicating antigen presence. Qualitative or quantitative measurement can be done based on colorimetric method. Fluorogenic substrates can measure the antigen concentrations more accurate and with higher sensitivity.

TYPES OF ELISA

Direct ELISA:

Direct ELISA is much faster and simpler than other ELISA techniques because fewer steps are required. In addition less mistakes are made due to fewer use of reagents. First, the antigen is attached to a plastic plate followed by another protein to block all other binding sites. In a separate reaction an enzyme is linked to an antibody. This enzyme-antibody complex is applied to the antigen. After the removal of excessive enzyme-antibody complex, the antigen bound to enzyme-antibody is left. Then the enzyme's substrate is added to get the desired color change.

Indirect ELISA:

The sample with the specific antigen which needs to be analyzed is attached to the wells of the microwell plate. Then a non-reacting protein solution is adhered to the wells to block the areas that are not coated with the antigen. A primary antibody, which binds specific to the antigen, and a secondary enzyme-conjugated antibody are following. A serum is then added to quantify the

primary antibody through a change of color. The concentration of the antibody correlates directly with the intensity of color.

Sandwich ELISA:

First, the well surface is coated with a known quantity of bound antibody to bind with desired antigen. Bovine serum albumin follows to block the nonspecific binding sites. Then the sample with the desired antigen is added to the microtiter plate. To „sandwich“ the antigen, a specific primary antibody is applied followed by enzyme-linked secondary antibodies that bind to the primary antibody. Antibody-enzyme conjugates that did not bind are then washed off. A substrate is added to get the desired color change to quantify the specific antigen.

The use of a purified specific antibody makes the need to purify the antigen from a mixture of other antigen unnecessary. That makes this analysis simpler and increases its sensitivity and specificity.

Competitive ELISA:

It starts with the incubation of the sample antigen with the primary antibody resulting in antibody-antigen complexes. These are attached to microtiter plate wells that are pre-coated with the same antigen. The unbound antibodies are washed off after an specific incubation period. The „competition“ takes place between the sample antigen and the antigen in the well: the more sample antigen bound to the antibody before given to the well, the less antigen can bind to the antibody coated on the wells. A secondary antibody with an enzyme is then added, followed by a substrate that leads to a fluorescent or chromogenic signal. If there is no color in a well, the presence of antigen in the sample is indicated.

Competitive ELISA provides a high sensitivity to differences in antigen mixtures. This is the main advantage of competitive ELISA. It is used for the measurement of allergen extracts, HIV antibodies or for example the total antibodies of the capsular polysaccharide of Haemophilus influenzae.

Portable and multiple ELISA:

This special form of ELISA uses a multicatcher device that contains 8 or 12 immunosorbent protruding pins on a stick. It can be dipped into the sample first and then into prefilled microwells containing reagents to perform the washing and incubation process. These ready to use ELISA kits are relatively cheap, can be used for a lot of samples and they don't need laboratory equipmet or skilled personnel.

They are used for detection of bacterial toxins, infectious diseases, oncologic markers or drug screening (Gan und Purtel, 2013).

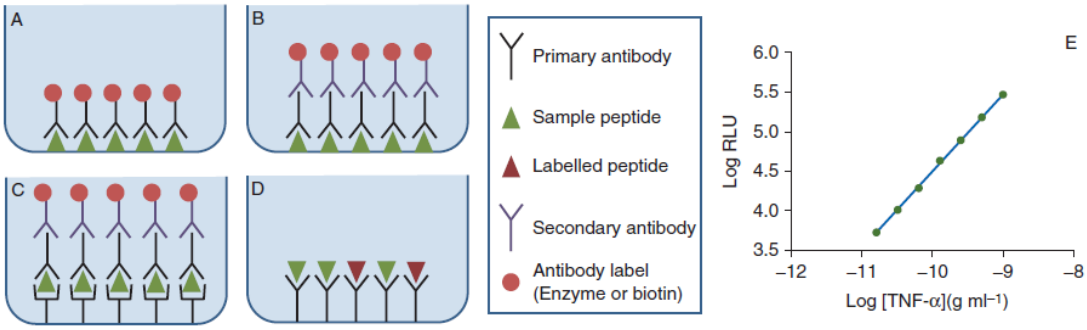

Figure 6: the differences of direct, indirect, sandwich and competitive ELISA (Grange et al., 2014)

Luminex®

In 1995 Luminex was incorporated and two years later the production of their first generation begun. The Luminex technology is based on paramagnetic microspheres, polystyrene or beads. The beads are dyed with red or infrared fluorophores in different intensities and each bead is given a number so the beads can be separated from another. Then the different bead sets are coated with a special antibody for a specific analysis. They can then be combined and put into a 96-well microplate to quantify and detect multiple targets at the same time (Dunbar and Li, 2010).

2.1.3 Measurement of Ghrelin in saliva

Most studies use blood or plasma samples to measure hormone levels in humans. But saliva has become important for the evaluation of pathological or physiological conditions. With better techniques and chemical instrumentation equipment, saliva-use for analysis has increased.

Saliva is a biological, watery fluid that is produced by major and minor salivary gland in the mouths of some animals, including humans. Saliva consists of 99% water, 0.3% proteins, 0.2% organic and 0.2% inorganic substances (Liu & Duan, 2012). Besides amylases, peroxidase, lipase, mucins, lysozyme, kallikreins, cystatins also growth factors and hormones are part of the organic components of saliva. It has many important functions such as lubrication, chewing, swallowing, digestion and perception of oral sensations. The estimated amount of daily salivary secretion is between 0.5 and 1.5 L (Kaczor-Urbanowicz et al., 2017).

Saliva plays a role in taste recognition and therefore in the gustatory system, which is important for food intake, determining food preferences and for energy, nutritive and electrolyte balance. Metabolic polypeptides in saliva like appetite and gut hormones (ghrelin, leptin, insulin and others) were considered to play an important role.

The usage of saliva as a biofluid brings many advantages. It is easy, fast, non-invasive and inexpensive. There is no need for needles, so anxiety levels can be reduced for patients. Salvia can be collected at home by an individual and there is no need for medical personal. The fluid does not clot and is easy to store and ship.

A major disadvantage nonetheless is the lack of suitable cost-effective technology. It needs a higher sensitivity and is technically more challenging. There is also a risk of contamination with topical hormones on hands or lips and an interference with beverages or food.

However, recent publications seem promising (Lima et al., 2009).

Salivary diagnostics can be used in many fields such as pharmacotherapy, medicine, epidemiology, dentistry or bioterrorism.
In medicine it is applied in oncology, auto immune diseases, cardiology or metabolic diseases. Another usage is the monitoring of poisoning or therapeutic drug levels (Kaczor-Urbanowicz et al., 2017).

Collection of saliva:

There are different methods of saliva collection: a swab is taken into the mouth, preferably under the tongue or in the cheek, to collect the saliva. The swab can consist of different materials such as polyethylene, cotton, polyester or cellulose. The swab has to be chewed for about 60 seconds to stimulate salivation. The swab is than placed into a plastic tube which is then centrifuged. (Gröschl et al., 2008)

In 2005 Gröschl et al. identified ghrelin in saliva and the 3 major salivary glands, therefore could verify the production of ghrelin by salivary glands. Oral keratinocytes and salivary glands produce both receptor isoforms of ghrelin (Gröschl et al., 2005). It is also found in the taste buds of the tongue. Ghrelin derives from a larger precursor by an enzyme (prohormone convertase 1/3) and both can be found in human taste cells. GOAT and GSHR can also be found in these cells. GHSR-null mice showed significantly lower taste responsivity to salty and sour tastants, which suggests that ghrelin has a role in salty and sour taste sensation (Fabian et al., 2015).

They also investigated if it is possible to detect both, acylated and non-acylated ghrelin. 7.8 ng/L was the lower detection limit for octanoyl-ghrelin. The hormone was measured with RIA from Linco Research. A significant linear correlation between plasma and salivary total ghrelin could be found, where plasma ghrelin levels being significantly higher than salivary ghrelin levels. Salivary and serum ghrelin ratios ranged between 1:1.5 and 1:8.
When lean and obese patients were compared, salivary values were higher in lean lean subjects.

Total ghrelin saliva ranged from 10 to 198 ng/L (Phoenix) and from 550 to 2470 ng/L (Linco), depending on the chosen RIA. The acylated form of ghrelin ranged from 55 to 451 ng/L with no correlation between total and acylated ghrelin from the same sample.
Salivary ghrelin is stable for up to 3 days in the refrigerator, but value decreases drastically at room temperature. Also, repeated freezing and thawing should be avoided.
Cross reactions with other salivary compounds are unlikely due to strictly linear serial dilutions.
The use of citric acid for stimulation of salivary flow rate could not be recommended due to decreased values.

Although statistical correlation between plasma and salivary ghrelin was too weak and the sample number too low in this publication, studies after that also measured salivary ghrelin (Gröschl et al., 2005).

2.2 Obesity

Obesity is a result between an imbalance between energy intake and expenditure. There was a massive increase in overweight and obese populations during the last decades. This fact is a major global health challenge and brings many health risks such as diabetes, high blood pressure, stroke, sleep apnea, cancer, asthma etc. (Ng et al., 2013).
For the fight against this major health problem ghrelin could be an easy and safe way of solution.

Ghrelin and obesity

The mechanism between ghrelin, hunger and obesity are still unclear.

There is a theory that ghrelin promotes gastric emptying and that this ends in an increase of hunger. Fasting ghrelin plasma levels are also correlated positively with gastric emptying (Tschop et al., 2001).
Compared to subjects with a healthy BMI postprandial suppression of ghrelin seems to be in obese subjects. This could lead to an increased food intake in obese patients because they still feel hungry after eating a meal (Camilleri et al., 2009).
The mean serum ghrelin level was reported lower in obese subjects in most studies (Dimitriadis et al., 2013; Camilleri et al., 2009).

Although there are already some studies investigating the influence of ghrelin on obesity there still needs to be more research about that topic because the mechanism is still unclear.

3. MATERIALS AND METHODS

3.1 Materials

The following databases were used for literature search: PubMed, Science Direct, and Embase. The literature search started at the beginning of March 2018 and lasted for 8 weeks, until May 2018. Inclusion and exclusion criteria were defined at the beginning and depending on them, search terms were selected. For each database the following terms were used: “ghrelin and ELISA”, “ghrelin and RIA”, “ghrelin and healthy”, “ghrelin and obese” and “ghrelin and analysis”.

Inclusion criteria:

- Human intervention
- Healthy and obese patients
- Patients had to fast before the measurement of ghrelin values
- Ghrelin values were measured in plasma, blood or saliva

Exclusion criteria:

- Children
- Weight loss surgery of any kind
- Administration of ghrelin or ghrelin mimetic before measurement
- Application of obesity induced medication
- Subjects with Prader - Willi syndrome
- Type 1 and 2 diabetes
- Ghrelin values measured in saliva

3.2 Methods

For this systematic review a total of 1083 hits were found during searching. First duplicates were removed and 876 hits remained. The filtration progress started based on the title and then hits were excluded if the abstract did not fit. Exclusion reasons were illnesses, language, medicine or ghrelin mimetic application, or surgeries. For some studies the full text wasn't available. After reading the texts, again some publications were excluded because of missing data or wrong study population. A very important inclusion criteria was that the study subjects had to fast before the blood or saliva sample was taken. Altogether, 30 studies were included in this systematic review. The studies were separated in 5 groups: healthy people measured with RIA, healthy people measured with ELISA, obese people measured with RIA, obese people measured with ELISA and ghrelin measurement in saliva. For the values measured in saliva only could be found so they were put in one group.

Translation into standard units

In order to compare the different studies it was necessary to convert all units of measure to pg/ml because it was used in most publications.

Some studies used fmol/ml and to convert that unit into pg/ml it is necessary to multiply with the conversion factor 3.372 (Ulasoglu et al., 2013).
If the unit used in the study is pmol/l the conversion factor is: pmol/l = pg/ml x 0.296 (molecular weight of ghrelin is 3.245) (Kim et al., 2005).

Figures and Tables

All data and consequently all figures and tables were created using Excel or word. If not lettered otherwise, all tables and figures were specifically created for this thesis.

4. RESULTS

4.1 General overview of analyzed data for values in blood and plasma

Altogether 1152 subjects were included for this part of the work. They were separated in subjects measured with ELISA, subjects measured with RIA and in these groups between healthy (non-obese) and obese subjects.

7 publications measured ghrelin in healthy subjects with RIA, 6 in obese subjects with RIA. 7 studies could be included for the measurement of ghrelin in healthy subjects with ELISA and 6 for analysis with ELISA in obese subjects.

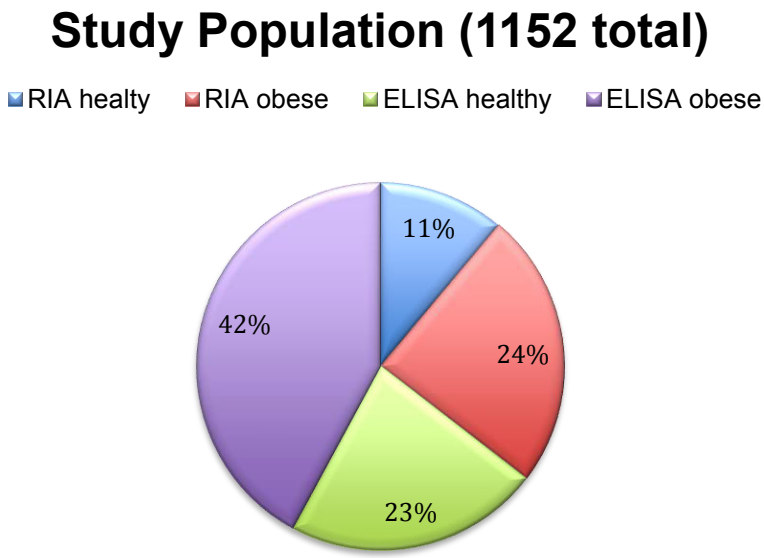

Figure 7: different methods of analysis in obese and healthy patients

All blood and plasma studies were performed between 2010 and 2018. To get a better comparison, saliva studies from all years were included. The group with the most subjects was the one including obese patients where ghrelin was measured with ELISA (n=485). For the healthy groups, control groups also were included.

For a better characterization of the groups the mean BMI and mean age was stated.

Study	BMI kg/m^2	Age in years
RIA healthy	23,5	38,6
RIA obese	40,2	40
ELISA healhty	22,5	38,5
ELISA obese	32,8	42,7

Table 1: BMI and age of all subjects

When it comes to the companies that produce ELISA and RIA kits there is a kit from one manufacturer that was used more often: Millipore (USA) was used in 13 publications out of 26 altogether. Especially studies that worked with RIA as method of analysis preferred Millipore (9 out of 13). Second most used manufacturer was Phoenix Pharmaceuticals (used 4 times).

Producers	used
Bioengineering Institute	1
Peninsula Laboratories	2
Millipore	13
Hosoda et al.	1
Sunlong Biotech	1
Phoenix Pharmaceuticals	4
Sangon	1
DRG	1
Bertin	1

Table 2: Producers of RIA and ELISA kits

4.2 Ghrelin values measured with RIA

In this chapter, different values of ghrelin in healthy obese and non obese subjects were compared. These studies were used because most ghrelin studies concentrate on obese and anorexic subjects. For the comparison of ghrelin values in healthy subjects control groups of studies were used.

All were measured with RIA which is besides ELISA one of the two most used method of analysis for ghrelin.

Ghrelin values measured with RIA in healthy subjects

Study population

7 publications met the research criteria. Altogether 128 (63 female and 65 male) subjects participated in the studies. Some studies only included healthy patients from the beginning, but also healthy control groups from other studies were included to get more data.

Inclusion and exclusion criteria for healthy participants

To be included here, healthy subjects had to meet some inclusion and exclusion criteria.
The criteria were as followed:

- no medical diseases or psychiatric illnesses (especially no history of eating disorders)
- no pregnancy or lactating
- no abuse of alcohol, tobacco or illegal drugs
- no current use of medications, which influence weight or appetite
- no history or clinical evidence of impaired fasting glucose or diabetes mellitus
- BMI had to be between 18.5 and 29.99 kg/m^2 (non-obese subjects)
- All studies had to be performed in 2010 or later

All participants had to be healthy because some illnesses have an influence on the ghrelin value. In addition, it was important that the subjects had no history of any eating disorder, a lot of studies focused on ghrelin values in patients with eating disorders and there were some differences in ghrelin values in people with and without eating disorders.

Author	Participiants	Ghre in pg/ml	Producer
Wang et al., 2014	38 (22m)	566.05	Nanjing Jiancheng Bioengineering Institute, China
Sanchez-de-la-torre et al., 2011	20 m	906.77	Peninsula Laboratories, USA
Purtell et al., 2011	10 (5m)	831	Millipore, USA
Yoh et al., 2010	A: 5m B: 5f	478.49 699.35	Developed by Hosoda et al.
Lawson et al., 2011	20 (f)	1049	Millipore, USA
Panek et al., 2014	20 (11m)	540,9	Millipore, USA
Panidis et al., 2010	10f	1,129.4	Millipore, USA

Table 3: Ghre values of healthy subjects measured with RIA

Comparison of Ghrelin values

As one can see in Table 3 the mean ghrelin values range from 478.49 to 1129.4 pg/ml (mean value was 775.1 pg/ml).

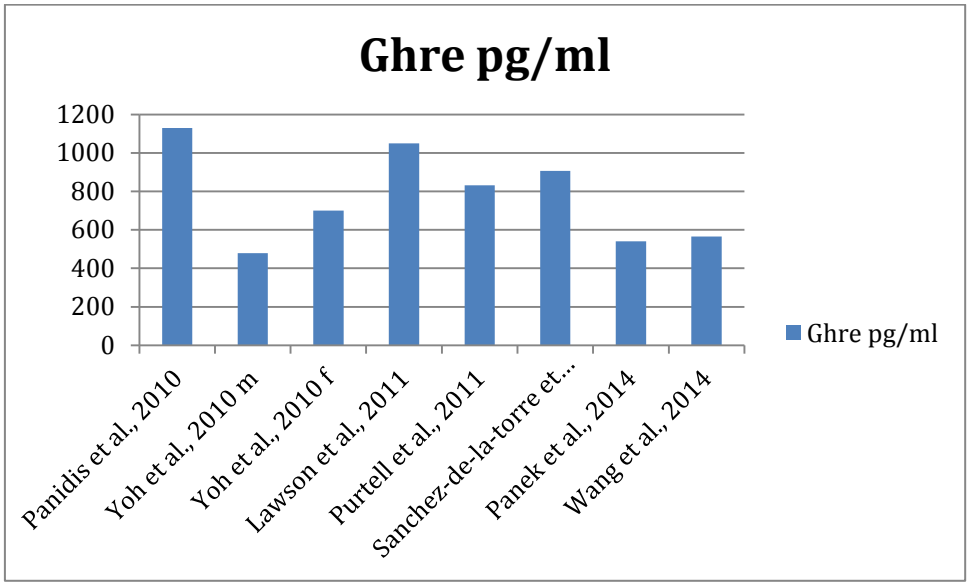

Figure 8: comparison of Ghre values of healthy subjects measured with RIA

It is not possible to compare the different values between genders because some studies only indicate a mean ghrelin value for male and female subjects combined. A reason for that could be that there was no significant difference between men and women in these studies although some studies have shown higher ghrelin values in female subjects compared to male subjects (Makovey et al., 2007; Soriano-Guillén et al., 2016). The only study that separated ghrelin values of male and female subjects was the one from Yoh et al. (2010). Here the women had higher ghrelin values in the mean, but the study population was relatively low (5 men and 5 women).

The mean age of participants is 38.6 years. The age could have a role in the difference of ghrelin values, which is discussed later in this work. The mean BMI here was 23.5 kg/m^2 which could also play a role in different ghrelin values.

Study	BMI kg/m^2	Age in yrs
Wang et al., 2014	22.5	53.3
Sanchez-de-la-torre et al., 2011	24.7	42.9
Purtell et al., 2011	21.4	28.9
Yoh et al., 2010	22.2	35.8
Lawson et al., 2011	22.3	27.2
Panek et al., 2014	27.1	52
Panidis et al., 2010	24.4	30
Average	**23.5**	**38.6**

Table 4: BMI and age of healthy subjects measured with RIA

Most studies used a RIA kit from Millipore, USA. The other RIA-kits were from Peninsula Laboratories (USA), Nanjing Jiancheng Bioengeneering Institute (China) and one kit was developed in-house from Hosoda et al. (2004).

Ghrelin values measured with RIA in obese subjects

Study population

Altogether 280 participants (124 male and 156 female) were found for comparison of values in obese subjects. All were measured with RIA.

Inclusion and exclusion criteria for obese subjects

Also, here the obese subjects had to meet criteria.
Criteria was:

- no medical diseases or psychiatric illnesses (especially no history of eating disorders)
- no pregnancy or lactating
- no abuse of alcohol, tobacco or illegal drugs
- no current use of medications, which influence weight or appetite

- no history or clinical evidence of impaired fasting glucose or diabetes mellitus
- BMI had to be over 30 kg/m^2 (no underweight or normal weight subjects)
- All studies had to be performed in 2010 or later

All participants had to be healthy besides being overweight.

Author	Participiants n (280)	Ghre in pg/ml	Producer
Tsoli et al., 2013	A: 12f B: 12f	Gr. A: 559 Gr. B: 639	Millipore, USA
Ramon et al., 2012	A: 7f B: 8f	584 610.2	Millipore, USA
Sanchez-de-la-torre et al., 2011	10m	733.3	Peninsula Laboratories, USA
Purtell et al., 2011	12(7m)	632	Millipore, USA
Lawson et al., 2011	17f	543	Millipore, USA
Crujeiras et al., 2010	162 (83m)	952	Millipore, USA
Average		**656.6**	

Table 5: Ghre values of obese subjects measured with RIA

Comparison of Ghrelin values

Ghrelin values in obese subjects were between 543 and 952 pg/ml (with the mean value of 656.6 pg/ml). Crujeiras and partners had by far the most subjects and also the highest mean ghrelin value. Because of the high number of subjects it is possible that this study is the most representative.

No study listed ghrelin values in male and female subjects separated so it is not possible to compare values of men and women.

Here the most used RIA kit was also from the producer Millipore (USA). Only one other study used a different kit from Peninsula Laboratories (USA).

Study	BMI kg/m^2	Age in yrs
Tsoli et al., 2013	A: 57.6 B: 43.7	42.3 40.3
Ramon et al., 2012	A: 44.2 B: 43.5	46.1 49.8
Sanchez-de-la-torre et al., 2011	32.1	48.7
Purtell et al., 2011	34.3	31.9
Lawson et al., 2011	31.3	29.5
Crujeiras et al., 2010	35	31.1
Average	**40.2**	**40.0**

Table 6: BMI and age of obese subjects measured with RIA

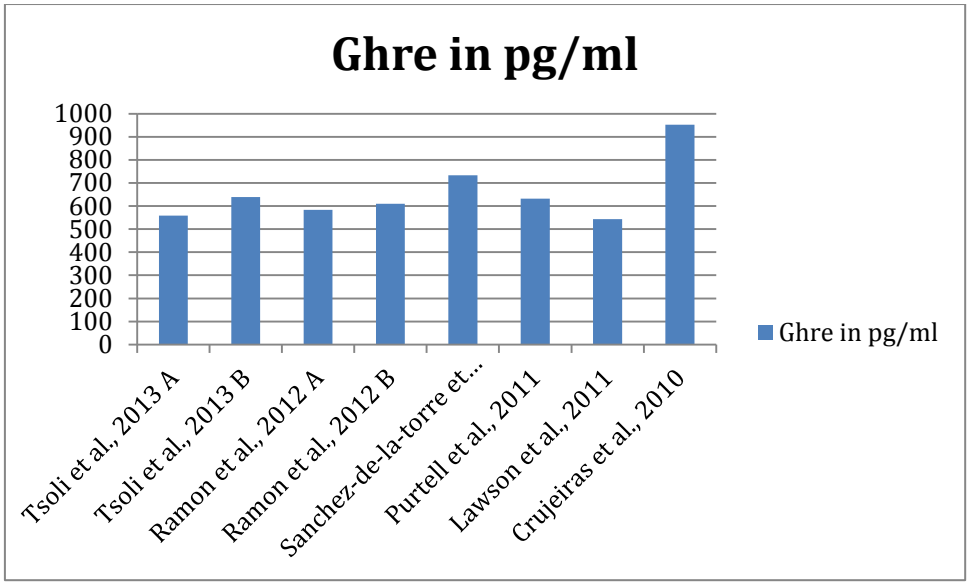

Figure 9: comparison of Ghre values of obese subjects measured with RIA

Comparison of Ghrelin values in obese and healthy subjects

The mean values of healthy and obese (775.1 and 656.6 pg/ml) were in similar range, hence it seems that the ghrelin values between healthy and obese subjects are relatively similar. There is a negative correlation between BMI and ghrelin levels which underlines the findings here. Also, ghrelin levels in obese subjects are lower after a meal compared to normal weight individuals (English et al., 2002).

Unfortunately, it is not possible to compare ghrelin values of different genders. There was no separation in different groups of gender.

4.3 Ghrelin values measured with ELISA

This chapter lays a focus on ghrelin values measured with ELISA. It is separated into two groups: measurement of healthy and obese subjects.

Ghrelin values measured with ELISA in healthy subjects

Study population

Altogether 259 subjects in 7 publications were included in this chapter. 102 subjects were female and 112 were male. Unfortunately Zhang et al. did not specify the percentage of men and women in their study.

The mean age of all studies was 38.5 years. Hamdy et al. couldn't be part of that calculation because they didn't state the mean age of their participants. Also only 4 studies could be used for calculation of the mean BMI (22.5 kg/m^2). All other studies didn't list the mean BMI of their study population (Hamdy et al, 2018; Zhang et al., 2015; Alver et al., 2014; Motawi et al., 2013).

Study	BMI	Age in yrs
Hamdy et al., 2018	n.a.	n.a.
Ucan et al., 2017	28.6	42.5
Zhi Jiang et al., 2017 Gr.A	20.6	24.1
Zhi Jiang et al., 2017 Gr.B	20.7	24.0
Zhang et al., 2015	n.a.	37.78
Alver et al., 2014	n.a.	51.26
Chen et al., 2013	22.25	67.1
Motawi et al., 2013	n.a.	52.6
Average	**22.5 (available for 4 studies)**	**38.5 (without Hamdy et al., 2013)**

Table 7: BMI and age of healthy subjects measured with ELISA

Autor	Participants	Ghrelin in pg/ml	Producer
Hamdy et al., 2018	10 (9m)	461.7	SunLong Biotech Co., LTD
Ucan et al., 2017	24 (6m)	44100	Phoenix Pharmaceuticals, USA
Zhi Jiang et al., 2017	A: 30 (21m) B: 10 (5m)	229.1 232.45	Millipore, USA
Zhang et al., 2015	45 (n.a.)	48840	Sangon Biotech, Shanghai, China
Alver et al., 2014	45 (17m)	6500	Phoenix Pharmaceuticals, USA
Chen et al., 2013	55 (30m)	392.3	Phoenix Pharmaceuticals, China
Motawi et al., 2013	40 (24m)	3370	DRG international,USA

Table 8: Ghre values of healthy subjects measured with ELISA

Inclusion and exclusion criteria for healthy participants

To be able to compare the ghrelin values measured with ELISA and RIA better, inclusion criteria was the same as in the chapter before:

- no medical diseases or psychiatric illnesses (especially no history of eating disorders)
- no pregnancy or lactating
- no abuse of alcohol, tobacco or illegal drugs
- no current use of medications, which influence weight or appetite
- no history or clinical evidence of impaired fasting glucose or diabetes mellitus
- BMI had to be between 18.5 and 29.99 kg/m^2 (no obese subjects)
- All studies had to be performed in 2010 or later

Unfortunately, the BMI was not listed in all studies but due to the fact that all subjects were listed as healthy it was assumed that their BMI was in a normal range.

Comparison of Ghrelin values

As one can see in figure 8 ghrelin values in different studies differ from one another to a great extent. They range from 229.1 to 48840pg/ml. This is a very wide range of values.

The work from Ucan et al. and Zhang et al. are showing very high ghrelin values (48840 and 44100 pg/ml) compared to the other publications. Zhang et al. used an ELISA kit from Sangon Biotech (Shanghai, China) with an assay range from 5000 – 160000 pg/ml (5 to 160 µg/L) which seems fairly high from the beginning. Unfortunately, there is no laboratory manual available for Sangons ELISA-kit. Also, there is no further description about the handling of blood samples or usage of anticoagulants, acids or other additives.

Ucan and coworkers used an ELISA kit from Phoenix Pharmaceuticals. The test range of the kit is 0- 100000pg/ml (0-100ng/ml) and their measured value falls into this range. In this study EDTA and aprotinin was used, which is recommended for the usage of samples for ghrelin measurement.

The Phoenix Pharmaceuticals ELISA kit was used 3 times, other studies used following producers: SunLong Biotech Co., Millipore, Sangon Biotech and DRG international.

The ghrelin values of the studies that used Phoenix Pharmaceutical kits were also very far apart (44100; 6500 and 392.3pg/ml).

Because of these very diverse ghrelin values, it is hard to compare the results in these studies. The high ghrelin levels are also varying strongly compared to the ones measured with RIA. This topic will be discussed later in this work.

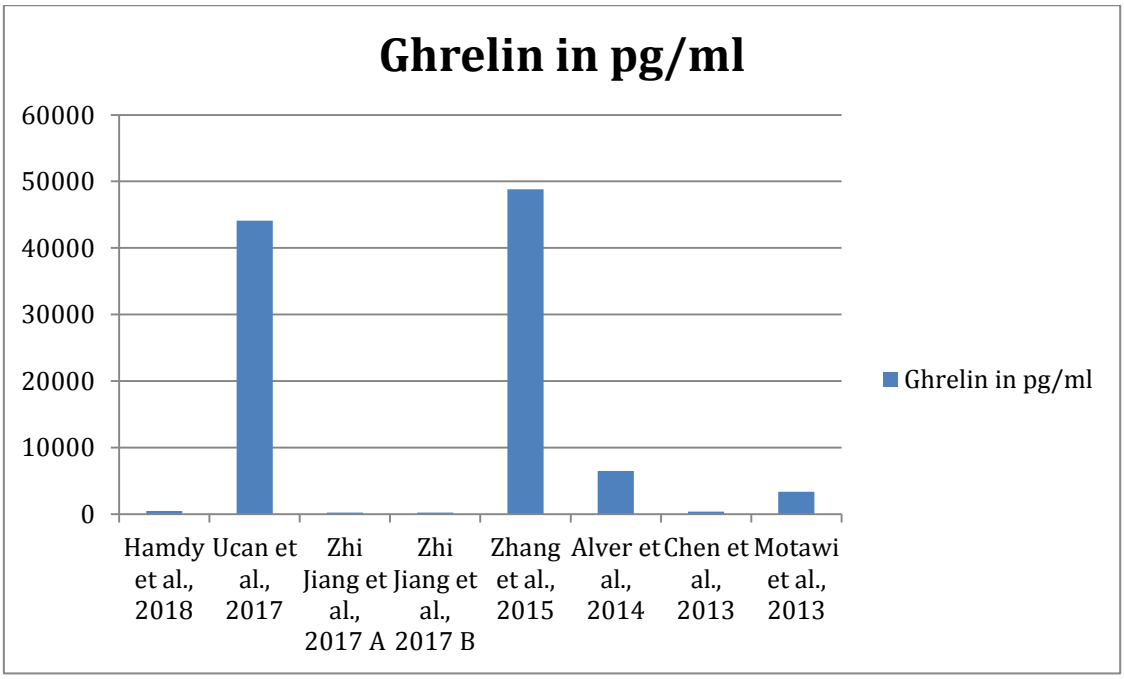

Figure 10: comparison of Ghre values of healthy subjects measured with ELISA

Ghrelin values measured with ELISA in obese subjects

Study population

485 subjects were found for this chapter of which 211 were male and 274 female. All subjects were obese and had a BMI of 30 kg/m^2 or more. The mean age of all studies was 42.7 years.

Author	Participants (n)	Ghre in pg/ml	Producer
Williams et al., 2016	A: 53 m B: 66 f	135.3 167.8	Bertin Pharma SPI-BIO, France
Hamed et al., 2011	30 (13m)	11970	Millipore, USA
Stepien et al., 2011	19 (4m)	1340.44	n.A.
Sofer et al., 2013	A: 30 (15m) B: 33 (17m)	707.54 707.53	Millipore, USA
Jakubowicz et al., 2013	A: 96 (39m) B: 97 (39m)	300.7 350.5	Phoenix Pharmaceuticals, USA
Ellis et al., 2012	A: 27 (13m) B: 34 (18m)	592 762.6	Millipore, USA
Average		**1703.4**	

Table 9: Ghre values of obese subjects measured with ELISA

Author	BMI kg/m^2	Age in yrs
Williams et al., 2016	A: 32.4 B: 31.6	43.6 41.7
Hamed et al., 2011	36.1	43.2
Stepien et al., 2011	33.2	53.0
Sofer et al., 2013	A: 34.2 B: 32.1	43 42.5
Jakubowicz et al., 2013	A: 32.2 B: 32.3	44.5 45.8
Ellis et al., 2012	A: 31.3 B: 33.0	34.3 35.8
Average	**32.8**	**42.7**

Table 10: BMI and age of obese subjects measured with ELISA

Inclusion and exclusion criteria for obese participants

To be able to compare the ghrelin values measured with ELISA and RIA better, inclusion criteria was the same as in the chapter before:

- no medical diseases or psychiatric illnesses (especially no history of eating disorders)
- no pregnancy or lactating
- no abuse of alcohol, tobacco or illegal drugs
- no current use of medications, which influence weight or appetite
- no history or clinical evidence of impaired fasting glucose or diabetes mellitus
- BMI had to be over 30 kg/m^2
- All studies had to be performed in 2010 or later

Comparison of Ghrelin values

It is clearly obvious in figure 7 that the measured ghrelin value of Hamed et al. was much higher (11970 pg/ml) compared to values measured in other publications. Also, the mean standard in this work is 8080pg/ml which means that the values have a wide range (5070 – 42700pg/ml). BMI in this study was slightly higher compared to the others, but this does not explain the much higher ghrelin value. In addition ghrelin values are negatively correlated with BMI (Purnell et al., 2013).

Mean age here was similar to the overall mean age of all studies. Hamed and coworkers used the ELISA kit from Millipore (USA) just like Ellis et al. and Sofer et al., but ghrelin values in these two works were much lower. In the laboratory manual for the usage of the Millipore ELISA kit the appropriate range of detection is 50pg/ml to 5,000pg/ml and that any result greater than that should be diluted (Sofer et al, 2013). Since all measured values from Hamed and coworkers are greater than that they probably have false values. Also, information is not provided if they performed their assay in duplicate or how the samples were treated (Hamed et al., 2011).

The study of Williams et al. had relatively low values compared to the other findings (135.3 and 167.8pg/ml), but the difference is not as extreme as the other extreme values of Ucan et al. (2017) and Zhang et al. (2015).

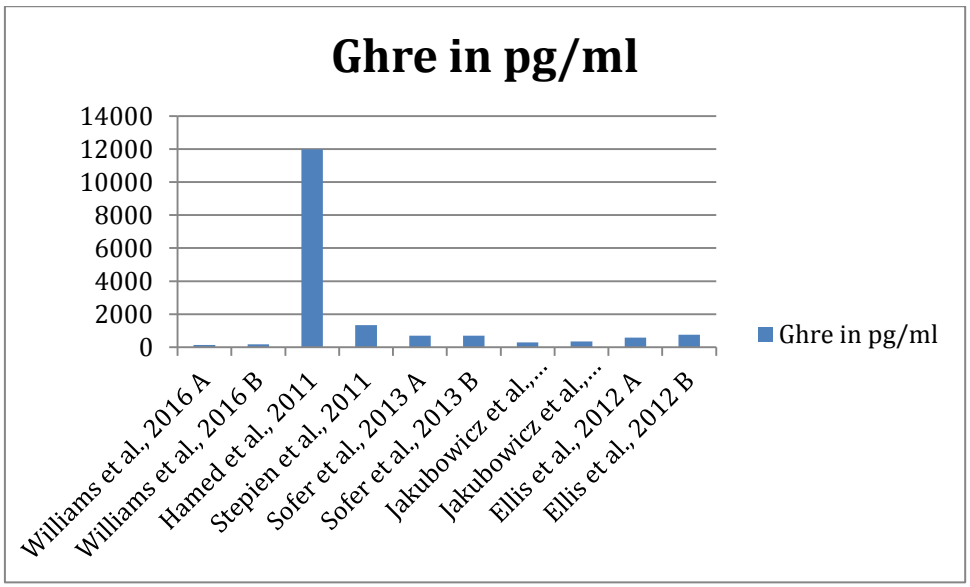

Table 11: comparison of Ghre values of obese subjects measured with ELISA

COMPARISON OF RIA AND ELISA METHODS

Ghrelin values measured with RIA had a range between 478.5pg/ml and 1129.4pg/ml where, on the other hand ghrelin values measured with ELISA had a very wide range of values: the lowest one was 135.3 pg/ml and the highest one was 48840 pg/ml.

These very different values make a comparison relatively hard. One problem with ELISA might be the lack of a well researched, standardized method. Some points that should be considered during the analysis of ghrelin with ELISA will be discussed in the latter chapter.

4.4 General overview of analyzed data for values in saliva

Study population

A few studies also used saliva for ghrelin analysis. Only four studies met the research criteria. In some publications ghrelin values were not stated. 101 participants were found altogether for the analysis of saliva. 70 male and 31 female subjects.

Author	Participiants n	Ghre in pg/ml	Producer
Kilic et al. 2017	30 (14m)	111.4	Linco Research
Kaabi & Khalifa 2014	30m	67000	TSZ-ELISA, USA
Aydin et al. 2005	25 (17m)	188.5	Phoenix, Europe
Cetinkaya et al. 2008	16 (9m)	87	Linco Research

Table 12: comparison of ghre values in saliva

Inclusion and exclusion criteria for analysis in saliva

The following criteria had to be fulfilled for ghrelin measurements in saliva:

- no medical diseases or psychiatric illnesses (especially no history of eating disorders)
- no pregnancy or lactating
- no abuse of alcohol, tobacco or illegal drugs
- no current use of medications, which influence weight or appetite
- no history or clinical evidence of impaired fasting glucose or diabetes mellitus
- BMI had to be lower than 30 kg/m^2

In addition publications before 2010 were included here to enable a better comparison.

Comparison of ghrelin values

Table 13 shows the different ghrelin values, which range from the minimum of 87 pg/ml to a maximum of 67000 pg/ml. The measured ghre value from the study of Kaabi & Khalifa is manifold higher compared to the other publications They stated that the assay was performed according to the manufacturers instruction (Kaabi and Khalifa 2014).. The used ELISA-kit that was not used in any other study, so the bid difference may be a result of different reagents or sample treatment.

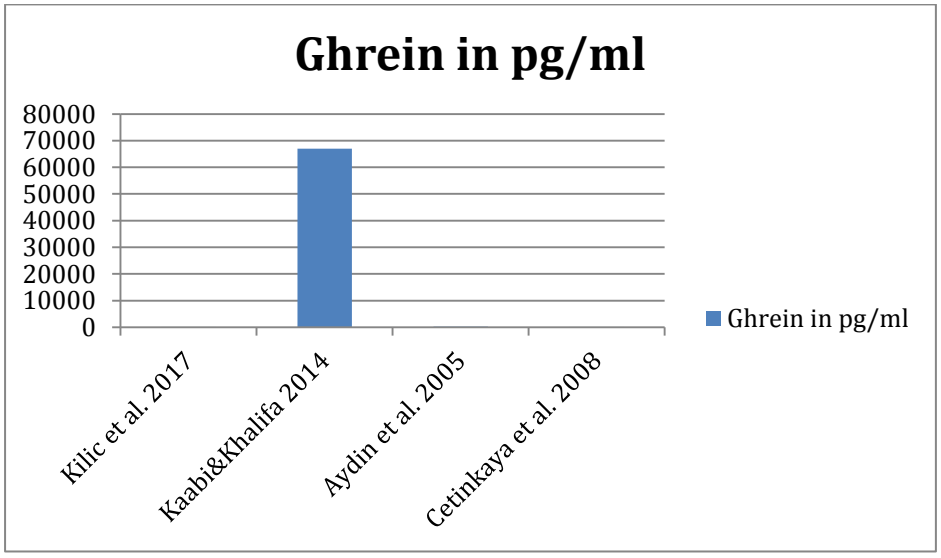

Figure 11: ghre values measured in saliva

ELISA was used in half of the studies, the other half used RIA.

As one can see in table 13 ghre values in saliva were higher compared to levels in serum in all but one study. This may be due to the fact that the salivary gland produces its own ghrelin.

Study	method	Ghre value in saliva in pg/ml	Ghre value in serum in pg/ml
Kilic et al. 2017	ELISA	111.4	205.8
Kaabi & Khalifa 2014	ELISA	67000	42000
Aydin et al. 2005	RIA	188.5	126.4
Cetinkaya et al., 2008	RIA	87	65

Table 13: comparison of ghre values in saliva and serum

Due to the very small size of studies and participants it is very hard to make a good statement about the analysis of ghrelin in saliva. For a better understanding of ghrelin in saliva more studies with a higher subject number have to be done.

5. DISCUSSION

As it can be seen in the discussed topics before, there are still some factors about ghrelin that need to be figured out. Especially when it comes to methods of analysis. Below different possible influences on ghrelin values will be discussed.

5.1 Possible influences on ghrelin values

As one can see ghrelin values in the selected studies are very different, especially those measured with ELISA. Particularly two publications stand out in the healthy ELISA group: Ucan et al. (2017) and Zhang et al. (2015). Both studies measured ghrelin values from over 40.000 pg/ml which is many times higher than the lowest value of 229.1pg/ml.
Kaabi & Khalifa (2014) also measured very high ghrelin values with ELISA in saliva and in serum.

There are different steps that should be considered during the analysis of hormones, in this case ghrelin. Some factors for the difficulty of having similar and comparable ghrelin values in analysis are discussed in the following chapters.

Methods and Producers

Different ELISA and RIA kits of different companies were used during the studies. In most publications a RIA or ELISA kit from Millipore (USA) was used. The RIA kit from this producer was used in nearly all chosen studies in 5 out of 6 studies in this work which makes a comparison possible. The mean values of these studies were in a range between 543 and 952pg/ml.

Besides Millipore only one kit was used more than once: the ELISA kit of Phoenix pharmaceuticals. Unfortunately, the measured values with this kit were so far apart that there is no comparison possible.

Most publications did not state if they followed the kit-manual and if they measured in duplicate. All companies recommend to measure samples at least in duplicate. It is also important to consider factors such as pH-value, temperature

and addition of anticoagulants or other additives that can help with the storage of ghrelin. These factors should also be listed in the kits manuals.

The influence on ghrelin levels during aging

Society is getting older and older and one worldwide medical goal is to achieve a long and healthy life. Aging is determined by environmental and genetic factors and a very complex, not fully researched process. During senescence a lot of changes are going on in the human body. The hormone balance changes in many ways, so maybe also human ghrelin levels. Ghrelin seems to play a role in many processes occurring during senescence such as anorexia, obesity or energy metabolism. It is not clear, if ghrelin levels change due to age dependent diseases or if the change in ghrelin levels plays a role in the upcoming of this disease. In some studies the administration of ghrelin seems to have a positive effect in elderly human subjects.

Aging is related with less appetite and energy intake, which is also called the anorexia of aging (Kojima et al., 1999). Ghrelin is an orexigenic peptide hormone, so it has been suggested that there is a link between the anorexia and ghrelin. Ghrelin also seems to have a role in aging.

Some early animal studies have shown an increase in ghrelin levels during aging, but others did not. Liu et al. found no difference in total ghrelin levels in serum between different ages of mice, but Kappeler et al. (2004) showed increased total ghrelin levels in two different kinds of rats. Also, studies with mice showed increased levels of acyl-ghrelin and total ghrelin in mice during aging (Akimoto et al., 2012; Sun et al. 2007).

Increased ghrelin levels through fasting were less robust in aging rats despite comparable basal ghrelin levels between 4 and 25 months of age (Wolden-Hanson, 2006). This could mean that aging impairs ghrelin expression in the gastric mucosa or that release of ghrelin is impaired in response to energy deficiency.

Many of the human studies have shown a reduction in circulating ghrelin between younger and elderly subjects. Rigamonti et al. showed a lower fasting ghrelin

level in elderly subjects (67-91 years) compared to younger control subjects (27-39 years) (2002).

Acyl-ghrelin is thought to stimulate growth hormone release and has orexigenic effects. Nass et al. could show a similar outcome with circulating acyl-ghrelin levels: 62-74 year old healthy male subjects had a lower level than 18-28 year old subjects (2014). Acyl-ghrelin was measured over 24 hours. The levels after meal intake were suppressed in both groups, independent of age. In addition, growth hormone levels were declined in elderly subjects. The elderly group consisted only of one woman, other participants were men but a study from Markovey et al. suggests that there is no significant difference between older men and women in circulating total ghrelin levels (2007).

Sturm et al. reported no difference between ghrelin levels in young and elderly female subjects (2003). The more recent study from Hickson et al. also could not find a difference in ghrelin or acyl-ghrelin levels between older and younger female subjects (2016). Schneider et al. compared malnourished and well-nourished young and elderly subjects (2008). They have shown an adaptive increase of ghrelin in the young malnourished study group, but no not in the malnourished elderly group. This could maybe explain the missing of compensatory hyperphagia after weight loss in elderly subjects.
In this study younger and elderly healthy subjects showed a similar ghrelin level .

There is also a change in the signaling of ghrelins GHS-R1a during aging. The treatment with elderly human subjects or animals with synthetic GHS-R1a agonists or ghrelin suggests that ghrelin signaling declines during aging. The decline is either a result of reduced GHS-R1a expression or a loss in its signaling (Yin and Zhang; 2015). The reason for the decrease of GHS-R1a signaling is associated with a reduction in parasympathostimulatory neuronal activity (Wu et al., 2009).

Energy metabolism, ghrelin and aging:

Energy metabolism changes during aging, so maybe ghrelin has a role in this alteration, but the reasons for changing in energy metabolism are various. A loss

of ghrelin activities rather than its plasma levels seemed to be linked with fewer food intake during senescence. Overnight fasting seems to have a stimulating effect on food intake in young but not in elderly mice. Also, the administration of ghrelin leads to higher food intake in younger but not in senescent mice (Akimoto et al., 2012). A study compared meal intake of GHS-R1a null mice and wild-type mice. The mice with deleted GHS-R1a showed increased food intake, but this outcome was less significant in mice aged 24-26 months of age. This outcome might show that deletion of GHS-R1a results in a changed meal pattern with senescence. The results of this publications suggest that there might be beneficial outcomes on metabolism during aging because of ghrelin receptor antagonism (Lin et al., 2014).

Other studies have shown a connection between ghrelin and age dependent bone loss, reduction of cardiovascular function, sarcopenia, neurodegeneration or adaptive immunity.

The change of ghrelin levels in most of the studies during aging should be considered during analysis.

The influence of different preparation techniques

Another important step in the process of ghrelin analysis is the preparation of ghrelin samples. Biological fluids consist of many lipids, proteins, salts and peptides hence there is a need to separate the different components of another.

Often choosing the right preparation step is more difficult than the analysis method itself. Separation of the analyte from the complex sample makes quantification and purification easier and saves time. One important and very common method for plasma clean-up and contaminant removal is the precipitation of proteins. For precipitation there should be three factors considered: what kind of contaminants or detergents have to be removed, whether the proteins must be kept in a denatured or native state; and the analysis which comes after the preparation step. There are different solutions that can be used for precipitation: acetone, trichloroacetic acid, chloroform-methanol, 2 propanol mixture or ethyl acetate. They all can remove contaminants. A 1:1 sulfosalicylic acid/acetonitrile solution showed the very good results: after shaking

and centrifugation (4°C, 3600 g) a clear supernatant remained (Rauh et al., 2007).

Sato and colleagues used traditional column chromatography for ghrelin extraction. The column provided three fractions. The downside of this method is the long time of analysis and the excessive usage of eluent (2005). Some other studies used mini columns (Rauh et al., 2007; Sidibé et al., 2014).

Solid phase extraction (SPE) is also used to extract ghrelin and proghrelin-derived peptides. SPE is used often for extraction, class fractionation, clean-up and pre-concentration of environmental pollutants. It is used for biological, clinical, beverages and food samples. The first usage of SPE was more than fifty years ago, but it has undergone a lot of changes during the decades. Part of SPE is the process of putting a solution onto a solid phase (a cartridge with the sorbent) which is able to absorb the target analytes, remove unwanted components and then elute the desired analytes using another solvent into a collection tube (Andrade-Eiroa et al., 2016). SPE is following two aims at the same time: pre-concentration and sample clean up. An often used cartridge for the extraction of proghrelin-derived peptides from the biological samples is Sep-Pak (Bang et al., 2007). It was used for fractionation before RIA for example (Hosoda et al., 2004). The negative side of this procedure is that it results in missing one quarter of ghrelin, most likely due to desacylation reaction (Bang et al., 2007). Therefore, the usage of polymeric SPE cartridges for ghrelin extraction is maybe a better option because these cartridges are more resistant to acidic pHs, so the usage of acidic plasma samples is possible.

One study used stir bar sorptive extraction (SBSE) as a preparation technique. For the sorptive extraction of the target compounds, a magnet bar coated with a sorbent polymer is used. This step is followed by analysis of the desorbed analytes such as HPLC/DAD, HPLC/UV, LC-MS and GC-MS. SBSE was used for the analysis of other hormones or drugs before. Eslami et al. (2016) could achieve great analytical performance with a linear range of quantification and good precision under certain circumstances. They recommend desorption time between 30 and 45 minutes, pH of 4 and no addition of salt.

It is also possible to use two or more of the pretreatment techniques, but this often takes a lot of time which could lead to des-acylation of ghrelin and raises the possible errors of quantification.

Chemical structure of ghrelin

Ghrelin is a peptide hormone which is made of 28 amino acids. It has a special modification on Ser3: it contains an O-acyl linked octanoyl group. It is the only known peptide with such modification. The modification is necessary for ghrelins activity. The human ghrelin gene is located on chromosome 3p25-p26. Human ghrelin genes contains 5 exons like the mouse ghrelin. Functional ghrelin peptide'S 28 amino acids are encoded in exons 1 and 2.
There is also a non-acylated form of ghrelin, des-acyl ghrelin, which can also be found in stomach and blood in significant levels. Food intake is also induced by des-acyl ghrelin.
Des-acyl ghrelin was long thought to be the inert degradation product of acylated ghrelin. It seems to have its own receptor and can also share the receptor with acylated ghrelin under experimental conditions. More studies show that des-acyl ghrelin is a functional inhibitor of acylated ghrelin. In obese human subjects with diabetes it can strongly suppress ghrelin. It can also improve postprandial glycemia and is said to be a potential drug for treatment of metabolic disorders or diseases where an elevated acylated ghrelin/des-acyl ghrelin ratio occurs like obesity, diabetes and Prader Willi syndrome (Delhanty et al., 2013).

The 10 amino acids in the NH2-termini are identical in many mammals, including human, mouse, rate, rhesus monkey, pig and cow. This suggests that this region is necessary for ghrelins activity (Sato et al., 2012).

The ester bond is enzymatically and chemically unstable, which can lead to the elimination of the octanoyl modification during handling, storage or dissolution in a culture medium. One way to get a more stable ghrelin structure is to add an acid such as HCl. Hosoda et al. experimented with different pH values (3,4,5,6 and 7.4). At 37°C ghrelin was most stable at a pH between 3&4. At pH between 3 and 5 and a temperature of 4°C ghrelins stability did not change significant after 6

hours. (Hosoda et al., 2004). Also Staes et al. tested the best storage conditions for ghrelin. In their study unacylated ghrelin was stable under all conditions, but the stability of acylated ghrelin suffered at 37°C at pH 7.4. Unacylated ghrelin is the major degradation product of acylated ghrelin. The reason for that is probably the saponification of the ester bound (2010).
Both studies suggest to store ghrelin at a low temperature and a low pH level.

There are esterase enzymes in human blood which may degrade the ester bond of octanoylated ghrelin. Hence it is also required to add esterase inhibitors like sodium fluoride (NaF), serine salicylate, phenylmethylsulfonyl fluoride (PMSF) etc. (De Vriese et al., 2004).
One study added PMSF as esterase inhibitor and HCl to human plasma samples and achieved ghrelin stability at room temperature for up to 3h (Sidibé et al., 2014).

A reason for the degradation of des-acyl ghrelin could also be its hydrolysis (Hosoda et al., 2004; Staes et al., 2010). De Vriese et al. (2004) discovered a major hydrolysis of the ester bond and a minor proteolysis (hydrolysis of peptide bond). Here, the addition of PMSF significantly reduced this reaction. The assessment of Trivedi and partners could not find a difference between the addition of PMSF and no addition of PMSF, but here this could also be a result of the very low amount added to the samples (2012).

Des-acyl ghrelin seems more stable than acylated ghrelin. Different storage conditions like temperature and pH value have less impact on the stability of des-acyl ghrelin (Hosoda et al., 2004). Rauh and partners worked with lithium heparin and EDTA. The addition of one of those substances results in production of desacyl-ghrelin. 11% of ghrelin could be found after 480 minutes of incubation with EDTA, des-acyl ghrelin on the other hand increased with time in a stochiometric ratio. With Li-heparin ghrelin desoctanoylated completely after 480 min and degradation of desacylghrelin was monitored. Proteolysis of des-acyl ghrelin was 4-5 times faster than hydrolysis of octanoylated ghrelin, this reaction was inhibited by EDTA but not by Li-heparin. The study suggests that the addition of EDTA is better for ghrelin measurement. They also added acetic acid to the plasma samples to achieve a lower pH level (2007).

Hosoda et al. used different anticoagulants with human blood samples: disodium EDTA with and without aprotinin, heparin sodium or no anticoagulant. The samples were incubated for different time spans at 4 or 37°C. Octanoyl ghrelin levels decreased significantly at the higher temperature especially the samples with no anticoagulants. EDTA with aprotinin turned out to be the best addition because decline of octanoylated ghrelin was less. Here a pH level between 3 and 4 was the best for molecule stabilization.
Hosoda and colleagues recommend blood collection with EDTA aprotinin, a fast centrifugation at low temperatures and a low pH (3-4) using HCl (2004). Staes et al. (2010) had similar findings considering temperature and pH levels.

Overall ghrelin samples should be collected into chilled tubes containing an anticoagulant, such as EDTA and aprotinin, should then be kept on ice at 4°C and should be mixed with an acid, such as HCl to adjust pH levels.

Protease Inhibitors

In many studies protease inhibitors (PIs) were used.

Protease inhibitors are molecules which inhibit the activity of proteases and hence stop the stop protein degradation. They can be small molecules, peptides or proteins. Protease inhibitors that occur naturally are often proteins or peptides themselves. They can be used as drug, during protein purification in vitro and in vivo.

There are varying mechanisms of protease inhibition. They are classified by the enzyme-inhibiting mechanism and the type of protease they inhibit. There are reversible and irreversible inhibitors. Reversible inhibitors bind to the protease to lower their activity or lower their speed. There is no reaction of the inhibitor itself. This group includes competitive inhibitors, non-competitive inhibitors and uncompetitive inhibitors.

Competitive inhibitors are competing with substrates over the active site residues. An example is aprotinin. Aprotinin is a competitive serine protease inhibitor. Aprotinin is often used during the sample preparation steps for ghrelin analysis.

Protease inhibitors can be purchased in cocktails with different types of protease inhibitors or individually. They are used for proteolytics assays of already purified proteins, theey can serve as a control or for protein purification. Commonly used PIs in experimental studies are AEBSF, Aprotinin, Bestatin, EDTA, Pepstatin, PMSF. AEBSF, Aprotinin and EDTA are also used for ghrelin analysis (Ritchie, 2013).

Most producers recommend the usage of PIs: Millipore recommends AEBSF and HCl, Phoenix recommends aprotinin usage, Bertin recommends PHMB, PMSF or Aprotinin and HCl. Some instructions and recommendations could not be found online.

PIs	function
Aprotinin	competitive serine protease inhibitor, reversible binding, forms stable complexes with active enzyme-site
AEBSF	Irreversible serine protease inhibitor, alternative to PMSF and DFP
PMSF	Non-specific inhibitor of proteases and other enzymes, irreversible, sulfonylates hydroxyl groups of the serine residues in the active site of enzymes

Table 14: most important PIs

The influence of nutritional state on ghrelin levels

Since ghrelin is involved in hunger and satiety its levels change due to nutritional state. It is not only important if and when someone has eaten before blood sampling, but also what was eaten. Some studies have shown different rise and fall of ghrelin levels after different intake of macronutrients. That is why it is very important to monitor the diet of human study subjects. Most of blood collection for ghrelin measurement is made after an overnight fast.

Already two years after the discovery of ghrelin Tschop et al. (2001) investigated the effect of meal intake on ghrelin levels in the human body. They wanted to

investigate the effect of ghrelin on gastric motor function, feedback signaling between nutrient intake and the central nervous system. Five healthy female and five healthy male volunteers were administered a meal after an overnight fast. Two hours after the meal, ghrelin levels were significantly lower than before. Also fasting plasma ghrelin levels were correlated with half-time gastric emptying. RIA was used for the measurement of plasma ghrelin (Phoenix pharmaceuticals, Inc., Belmont, CA). The RIA-kit uses a 125I-labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against full length, octanoylated human ghrelin. RIA was used because the reliability was confirmed before with rat plasma ghrelin levels (Tschop et al., 2000).
A finding of that study was that individuals with high basal ghrelin levels shows slower gastric emptying and the other way around. Tschop and colleagues suggested that ghrelin has a role as a signal that informs central regulatory systems to save energy by shifting to a different metabolic rate.

Cummings et al. also researched the role of ghrelin and food intake in the same year (2001). 10 healthy subjects (1 man and 9 women) participated in the study. They were given meals at 8am, 12am and 5:30pm. Blood was drawn direct into EDTA tubes every 30 minutes. Samples were stored at 4°C directly after the blood take and centrifuged afterwards. Plasma was stored until further investigations at -70°C. Cummings used the same RIA-kit for measurement of ghrelin. Lower and upper detection limits were 80 and 2,500 pg/ml. Ghrelin levels rose about 78% 1-2 h before the beginning of each meal and fell below fasting levels one hour after the meal was consumed.

One subject had very low ghrelin levels compared to the others: 24-h AUC 3,073 and 4.121 pg day/ml for the low-ghrelin subject vs. an average of 14.222 pg-day/ml for all other subjects. Cummings supported their thesis that ghrelin plays a role in meal initiation.

One study investigated different durations of fasting periods on ghrelin levels.
Nine healthy men ate a standardized meal after a 12 or 17h fasting period.
Plasma immunoreactive ghrelin was measured by radioimmunoassay kit
(Phoenix Pharmaceuticals, Belmont, CA). There were significant higher levels of ghrelin after the 17h fasting period compared to the 12h fasting (160±20 vs. 146±18 fmol/mL, P=0.015). There was no significant difference in the AUC

between the 12h and 17h fasting group in the decrease of ghrelin levels after the consumption of the standardized meal.
This suggests that the duration of the fast also could make a difference in ghrelin levels (Briatore et al., 2006).

The Study from Olabi et al. focused on the difference between the acceptance of a certain meal and ghrelin levels in humans (2018). 11 healthy men participated in the study and blood samples were taken after a 12h fast. Acylated ghrelin levels were measured with ELISA (enzyme-linked immunosorbent assay kits, Millipore Corporation, St Charles, MO, USA). The focus in this study was on the difference between ghrelin levels after one low and one high acceptability meal. It consists of vanilla custard with acesulfam K or without it. After both meals ghrelin levels in the subjects decrease after 15, 30 and 60 minutes and then increase after 90 until 240 minutes. A significant difference in ghrelin levels were shown after 180 and 240 minutes, here the values for the low acceptability meal were higher. Ghrelin levels started at approx.310 pg/ml for both groups, decreased to approx.200 pg/ml and then rose again. After 180 minutes ghrelin for the low acceptability meal was at approximately 300 pg/ml and for the high acceptability meal at about 360 pg/ml. At the end of blood sampling (240 min) ghrelin was about approx.360 pg/ml for high acceptability meal and approx.450 pg/ml for the low acceptability meal. The results of this study suggest that ingredients of a meal could affect appetite and food intake through stimulation of hormones like ghrelin.

A study looked deeper into the difference of postprandial levels of ghrelin after different meal contents: subjects ate a high fat (HF), high protein (HP), high carbohydrate (HC) or mixed meal. The study included 9 lean insulin-sensitive men (mean±SEM HOMA-IR 0.83±0.10) and 9 young obese insulin-resistant men (HOMA-IR 4.34±0.41). Blood samples were collected after a 10h fasting period and then after 30, 60, 90, 120, 180 and 360 minutes after meal-intake.
For the measurement of plasma acyl-ghrelin, venous blood samples were collected into EDTA Na2-containing tubes, half a tablet of EDTA-free Protease inhibitor and 0.5mg DPP-4 inhibitor. (Samples were centrifugated at 3000xg; for

15 min at 4°C, plasma was separated and stored at -80°C until further use for analysis).

Acylated ghrelin was measured with ELISA (Millipore, USA) in duplicate. The fasting ghrelin values were 313.3±60.8pg/ml for lean test subjects and 188.8±30.9 pg/ml for obese subjects.

Between lean and obese subjects the overall mean postprandial responses for ghrelin were not statistically different. The HF meal resulted in a greater suppression of ghrelin compared to the HC meal. The HP meal induced lower postprandial response compared to the HC meal. The postprandial curves for ghrelin were smaller after HF or HP meal compared to HC meal in obese subjects but not in lean subjects.

After all three meals, the postprandial ghrelin suppression was very similar in lean subjects, some other studies have shown that high protein meals suppress more ghrelin postprandial than high fat or high carbohydrate meals (Parvaresh et al., 2018). In the study from Foster-Schubert et al. (2008) 16 healthy subjects consumed different liquid meals containing either 80% protein, carbohydrates or lipids. Blood samples were collected after a 12 hour fast and put in a chilled tube containing 1.8 mg/ml EDTA and 4 mM 4-(2-aminoethyl)-benzene sulfonylfluoride. Acyl-ghrelin was then measured with ELISA which they developed themselves and total ghrelin was measured with RIA (LINCO Research, Inc., St. Charles, MO). Fasting total ghrelin was 786 ±246, 768 ±290 and 756 ±266pg/ml in all three groups and acyl ghrelin was 98 ±75, 102 ±74, 101 ±81pg/ml. Acyl-ghrelin decreased in all groups after consumption of the beverage: in the carbohydrate group levels decreased 34 ± 25 pg/ml, in the protein group 38 ± 32 pg/ml and in the lipid group 38 ± 28 pg/ml. Total ghrelin levels decreased 537 ± 151 pg/ml in the carbohydrate group, 562 ± 239 pg/ml in the protein group, and 590 ± 209 pg/ml in the lipid group. This study showed a greater suppress of total ghrelin after consumption of lipids compared to proteins and carbohydrates. Another finding was that only in the carbohydrate group was a raise of ghrelin above the initial preprandial ghrelin levels after three hours. This could mean that hunger returns earlier after a consumption of a carbohydrate rich food. Tannous et al. (2006) also showed longer low levels of acylated ghrelin after a high protein meal compared with high carbohydrate or high fat meals.

In obese subjects a high fat or high protein meal suppressed ghrelin better than a high carbohydrate meal postprandial in contrast to other studies.

Frequently made mistakes during analysis

Another part that should be considered are that mistakes can be made in the laboratory from the research-crew. Frequently made mistakes are the following:

- It is crucial to check the producer manual and the different reagents for storage temperature.
- Most reagents need to be at room temperature before using, so the should sit on bench for 15-20 minutes before using.
- Chemical reagents and kits have an expiration date that should be checked before using in an analysis.
- The producer manual has to be studied precisely and be followed correctly, all values have to be converted in the same unit.
- Pipetting technique has to be precise and all calculations should be double checked.
- Tips have to be changed if used for a different sample or another concentration.
- If necessary, samples have to be diluted (if levels are out of detection range).
- During the wash-phase of the wells it is important to be careful to not touch the coated well with the pipette. If an automated washing machine is used it has to be calibrated before.
- After the washing step any residual fluid has to be removed completely.
- The plate reader has to be set accurately.

It can be seen that there are a lot of steps during analysis where mistakes can be made. Therefore, it is essential to follow each step with care (Crowther, 2009).

5.2 Limitations and Strengths

There are some limitations and strengths of this work. Unfortunately one limitation is that not all studies could be included due to lack of access. These publications could be of big interest and could contribute to new findings.

Another limitation of all publications is that only total ghrelin is measured. This fact fails to differentiate between the different forms of ghrelin (acylated and non-acylated). Also some studies didn't state at all if total, desacyl or acylated ghrelin was measured which makes comparison impossible.

A lot publications had relatively small amounts of study populations, which makes it hard to say if the outcomes are significant.

Regarding the sample procedure the exact process is often not stated in studies.

6. CONCLUSION

Ghrelin was discovered in 1999. Since then a lot of different studies were made. Studies concentrated most on obesity, eating disorders, appetite and hunger metabolism. But over the years also findings between cancer, sleep/wake rhythm, sleep apnea and other diseases and body functions where made.

Ghrelin application was also seen as a promising therapy method for some diseases, especially those related to hunger and satiety. There are still a lot of open questions about the exact function of ghrelin in the body, those definitely have to be investigated before ghrelin can be used as a therapy method.

A really important part of the investigation of ghrelin is the method of analysis. Unfortunately it seems like some methods of analysis are not used in the same way. To have compareable results from different studies it is very important to have a standardized method for the measurement of ghrelin. Therefore it is necessary to consider all the steps that lead to the measurement of ghrelin. It starts with the age, weight and nutritional state of subjects. Then one have to consider which material is used for blood sampling, which additives are added, which pH-value is measured and at which temperature the sample is stored.

Hence, it is really important to invent a standardized method of ghrelin measurement. Then it is possible to investigate the potentially positive characteristics better and to get a potential therapy for diseases where ghrelin is involved.

LIST OF FIGURES

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7. LITERATURVERZEICHNIS

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