Measurement of Gastrointestinal Hormones

Problems, Pitfalls and Solutions

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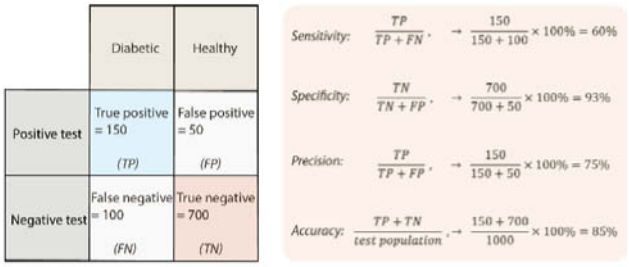
ABBREVIATIONS

CLSI = Clinical & Laboratory Standards Institute
PG 1-61 = Proglucagon 1-61
DPP-4 = Dipeptidyl Peptidase 4
RYGB = Roux-En-Y Gastric Bypass Surgery
EDTA = Ethylenediaminetetraacetic acid
RIA = Radioimmunoassay
ELISA = Enzyme-linked Immunosorbent Assay
T2DM = Type 2 Diabetes Mellitus
FDA = U.S. Food & Drug Administration
GCGR = Glucagon receptor
GFR = Glomerular Filtration Rate
GLP-1 = Glucagon-like Petide-1
GLP-1R = GLP-1 receptor
HPLC = High Pressure/Performance Liquid Chromatography
nAUC = Net Area Under the Curve
OXM = Oxyntomodulin
PC1/3 = Prohormone Convertase 1/3
PC2 = Prohormone Convertase 2
PCA = Principal Component Analysis

BACKGROUND

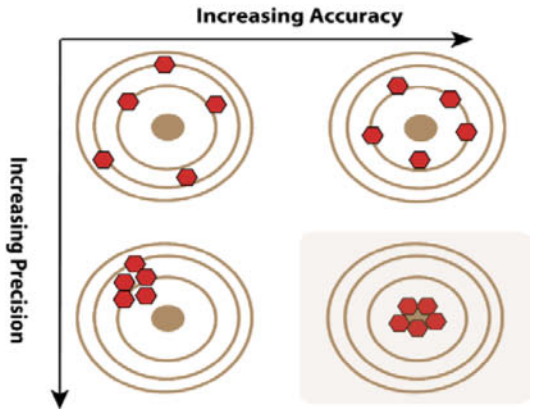
Since the times of the ancient Greeks, it has been known that the composition of blood not only reflects health status but as well and more importantly, disease. Biomedicine and clinical medicine have exploited the many chemical signals in our circulation to understand the physiology of man and the pathophysiology of disease, and the results have been implemented for clinical diagnostics and for the development of new drug targets. Diagnosis of disease includes several strata, including anamnesis, clinical findings, imaging, pathology and clinical biochemistry. The interest in blood borne markers has grown considerably, and the number of FDA (U.S Food & Drug Administration) approved biomarkers has exploded during the last decades. The major challenges in analytical biochemistry relate to fulfillment of the four classical reliability criteria: sensitivity, specificity, precision and accuracy [1, 2]. In clinical medicine, the same four topics are evaluated using the classical binary classification test (dichotomous outcome: Yes or No) [3, 4]. To illustrate this, let's imagine a scenario where 1000 subjects are tested using a new method to diagnose diabetes:



The figure above illustrates a scenario where 1000 subjects, hereof 250 with diabetes and 750 without, are tested using a new method to diagnose diabetes. The four classical criteria may be calculated as shown at the right panel. Precision may also be termed positive predictive value. Other concepts, such as negative predictive value and kappa value, may be included in the evaluation of a new diagnostic test [5].

Whereas this approach may be useful for studies investigating the potential benefits of new diagnostic methods, it does not necessarily fit in analytical biochemistry evaluating new methods assessing the secretory profiles of plasma proteins and peptides. Here, evaluations generally follow guidelines, for instance those of FDA, Clinical & Laboratory Standards Institute (CLSI), which include standard protocols for the assessment and evaluation of new and established immune-based detection methods for peptides circulating at low concentrations (low-abundant peptides). We have developed, evaluated and applied such approaches as described in

several publications, not included in this thesis but supporting the studies presented here [5, 6]. In general, the interpretation of the terms precision and accuracy, used in the subsequent studies, is closely related to (one may say identical) with that usually used in clinical medicine, and can be illustrated by the classical bull's eye approach shown below:



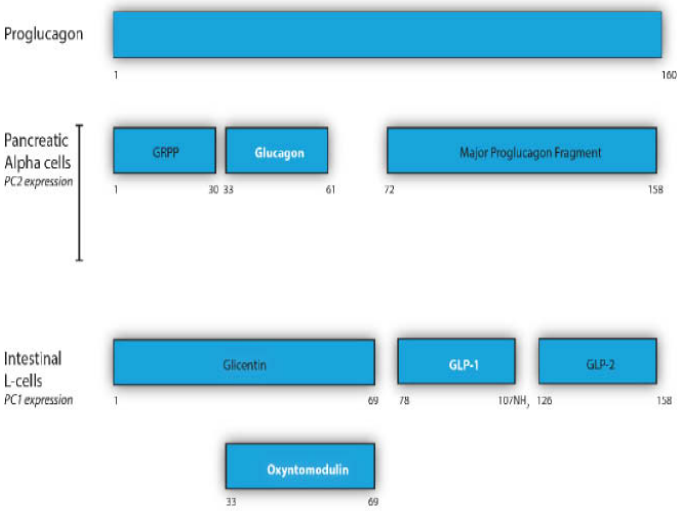
The figure above illustrates the concept of precision and accuracy. Whereas precision relates to 'how sufficient' the method of interest is to determine the concentrations measured from the sample, accuracy defines 'how sufficient' the method is to determine the 'true' concentration in a sample set. In the lower right quadrant, optimal conditions including sufficient accuracy and precision, of an analytical method are shown.

THE RATIONALE

Metabolic diseases, including morbid obesity and diabetes, affect millions of humans, severely impair quality of life [7] and increase mortality rates [8]. Accurate measurement of low-abundant peptides, such as several gut hormones, has helped identifying a number of novel drug targets for glucose-lowering therapy and weight loss [9], including receptor agonists/antagonists and inhibitors of the enzymes involved in processing and metabolism of the hormones. Good examples include insulin-stimulating factors such as the gut hormone, glucagon-like peptide-1 (GLP-1). Clearly, knowledge of the physiology, and in particular the pathophysiology, of such gut hormones is essential for the development of therapeutics based on these hormones, and this relies on the ability to accurately measure established as well as less established regulatory peptides.

Introduction to Gut Hormones

In the 1970s, a new field in metabolism emerged [10], investigating whether glucose homeostasis, appetite and metabolism are controlled by factors secreted from our gastrointestinal tract. It soon became clear that cross-talk between the gut, the pancreas and the brain may be mediated by peptide hormones secreted from the gut [11] that subsequently interact with G-protein coupled receptors expressed in several tissues [12] including the pancreas and neural tissues. These gut-derived signals, termed gut hormones, are secreted in response to a variety of nutrients. Some of them are involved glucose metabolism and play roles in the pathogenesis of diabetes; indeed, receptor agonists mimicking the native peptide hormones but with improved pharmacokinetics are now used in the treatment of several diseases, including but not limited to type 2 diabetes [13] and obesity [14]. Three of these hormones, glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM) and glucagon are of substantial interest particularly in relation to metabolic diseases [15]. These three peptide hormones are processed from the same precursor molecule (proglucagon):



The figure above illustrates the processing and splice variants of proglucagon in pancreas and the intestine. In pancreatic alpha-cells proglucagon is, due to expression of prohormone convertase 2 (PC2), spliced to glucagon, glicentin-related polypeptide (GRPP) and Major Proglucagon Fragment. In contrast, in intestinal L-cells, proglucagon, due to the expression of prohormone convertase 1/3 (PC1/3), forms glicentin, glucagon-like peptide-1 (GLP-1) and 2 (GLP-2), and oxyntomodulin.

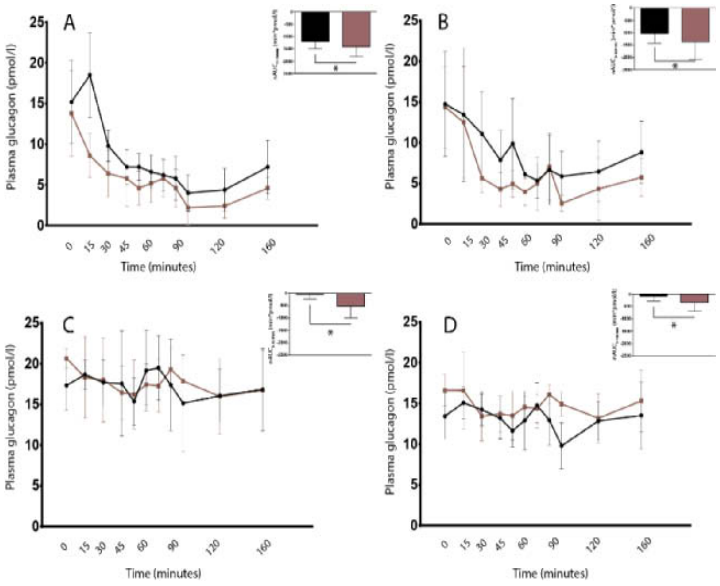
Whereas GLP-1 and OXM are secreted from the gut, glucagon is secreted from the pancreatic alpha-cells [16]. GLP-1 and glucagon bind their cognate receptors (with small overlap), whereas a specific receptor for OXM has not been identified so far; however, OXM binds to both the glucagon receptor (GCGR) and the GLP-1

receptor (GLP-1R) [17]. Interestingly, GLP-1 and glucagon may have opposite effects on glucose metabolism, explaining why agonists and antagonists, respectively, of these receptors are of keen interest in glucose lowering therapy in humans.

ACCURATE MEASUREMENT OF GUT HORMONES – A TRICKY BUT CRUCIAL PART

Measurement of peptides is not a *'new thing'*. In the late 1950s in a Veterans Administration hospital in Bronx, New York, the two pioneers of the radioimmunoassay (RIA), Rosalyn Yalow and Solomon A. Berson, used radioactively-labelled peptides to study diabetes (they were interested to know what happens to the insulin molecule once injected into the human body) [18]. Later, Yalow received both the Nobel price and Lasker award for the development of RIA. Since that time, both the RIA methodology (shifting from the use of iodine 131 to iodine 125) and the entire field of immune-based measurement of gut hormones have changed significantly, but similar analytical challenges still apply to modern techniques. A major advance in technology was generated by the two Swedish scientists, Peter Perlmann and Eva Engvall, who developed the enzyme-linked immunoassay (ELISA) [19]. The ELISA technology has become one of the most applied methods for peptide and protein measurements. Whereas, the RIA is competitive as standard, the ELISA may not necessarily be. A further important methodological improvement was introduced with the sandwich techniques, referring to the combined use of a capture and detection antibody (with only the latter coupled to some sort of enzymatic detection, for example horseradish peroxidase). The conceptual idea of an ELISA has evolved to include multiplexing methods that allow simultaneous detection of multiple analytes (most use 96 or 384 well plate layouts). A variety of detection approaches has been developed, all with the promise of generating improved sensitivity; these include fluorescent (Luminex Technology), laser (also Luminex), and electrochemiluminescence (MSD: Meso Scale Discovery) detection methods. To my disappointment, none of the current methods actually have improved sensitivity, but instead, seem to add further complexity to accuracy issue due to inadequate specificity – as has become apparent with assays for GLP-1, glucagon and OXM [5, 6].

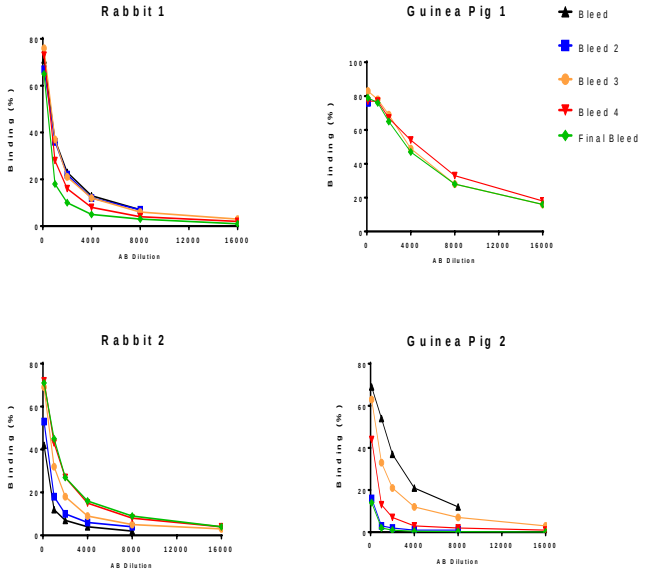
One may ask why it is important to accurately measure plasma concentrations of gut hormones - The obvious answer to that is that without accurate measurement, we are unable to discriminate between a clinically relevant finding (for this case, inhibition of glucagon secretion) and *a random analytical error*. To illustrate this, we assessed plasma glucagon concentrations in 5 healthy subjects that were clamped at blood glucose concentrations of 6mM [20]. Our results (see below) show that if researchers choose to estimate glucagon levels using the methods depicted in C (Luminex) or D (MSD) compared to A (RIA) and B (ELISA), they will not be able to identify a fundamental aspect of glucagon physiology: the glucose-induced inhibition of glucagon secretion from the pancreatic alpha-cell [21].



Plasma glucagon levels in five healthy participants during a 6 mM glucose clamp with simultaneous infusion of either saline (black) or atropine (red). (A) depicts a radioimmunoassay (codename 4305); (B) depicts a standard ELISA; (C) a chemiluminescence based ELISA; and (D) depicts a homogeneous time-resolved fluorescence based ELISA. Net area under the curve (nAUC) is depicted separately at upper right quadrant on (A), (B), (C), and (D). ∗represents a significant two-sided t-test comparing saline nAUC to atropine nAUC. Data illustrated as mean ± standard deviation. Adapted with permission from [20].

The underlying explanation for the failures in the above example is complex and may include several important components in analytical biochemistry. One obvious cause may be related to the choice of the antibody. In the current case for glucagon and its suppression during glucose infusion, a number of C-terminally directed antibodies have been developed including the ones used in the Holst laboratory (code name 4305: [22]); so far most have been polyclonal. By applying a C-terminally directed antibody, this will provide

the assay with a certain specificity, because the antibody ideally will not be able to react with C-terminally elongated forms and therefore will not react with glicentin and OXM (but of course this has to be tested). When designing sandwich ELISAs, targeting both termini of glucagon, a problem with generating suitable N-terminal antibodies typically appears. From 2011 to 2014, we generated N-terminal antibodies (titer-curves of four of the 24 antibodies are shown below) from an in-house production, using rabbits and guinea pigs immunized with an N-terminal fragment coupled to Keyhole Limpet Hemocyanin and from external companies that used a variety of techniques including phage-display technology.



The figure above shows 4 panels, each representing the binding (percentage) of an antibody raised against the N-terminus of OXM/glucagon (as they have identical sequence of their N-terminus). Serum obtained at different time points (Bleed, Bleed 2, Bleed 3, Bleed 4 and Final Bleed) during the immunization process are shown in different colors in each panel.

The only one that in fact worked, i.e. did not cross-react with other proglucagon molecules, was produced in-house in guinea pigs, but its affinity (and therefore sensitivity) was not sufficient to allow its use in an in-house sandwich ELISA for either glucagon or OXM. A global internet search for suitable N-terminal glucagon antibodies was also unsuccessful. This example also demonstrates potential differences in the immunogenity of different amino acids sequences, and highlights the importance of specificity. Generating polyclonal antibodies has the advantages that they are fairly easy to make, but they recognize multiple epitopes on the antigen (probably contributing to their high affinity), whereas monoclonal antibodies solely recognize a single epitope, but are extremely laboursome, expensive, and require special technology. As we later will demonstrate (**Study 2**), we did in fact co-develop specific glucagon and OXM sandwich ELISAs for the use in humans, and later for rodents [23], using monoclonal mouse antibodies.

Summary and Discussion of the Main Results

Considerable interest in proglucagon-derived peptides and their role in biology has arisen over the last decades, and this might have to do with their applicability for anti-obesity and anti-diabetic drug development, the so-called GLP-1R analogues and DPP-4 (dipeptidyl peptidase-4) inhibitors. Not only has there been enormous focus on GLP-1, but a growing interest in other proglucagon-derived peptides, in particular glucagon and OXM, has come. Studies have mostly investigated the potentiality for drug development from these hormones, but still we don't really know that much about them. A main reason for this is that accurate measurements have not been available.

In the following studies, we first investigated the pre-analytical and analytical sample handling in a systematic manner to generate and provide the community with a sort of guidelines (caveats) for measuring GLP-1 and glucagon in humans (**Study 1**). Secondly, we developed a mass-spectrometry based method that enables scientists to identify known and unknown hormones such as OXM and to validate the accuracy of the ELISAs used for measurement of them in clinical studies (**Study 2**), and finally we applied the methods and knowledge to investigate the molecular heterogeneity of glucagon in diseases where it is hypersecreted (**Study 3**) and, as a consequence, we identified a new glucagon variant in humans with glucose-regulating capabilities (**Study 4**).

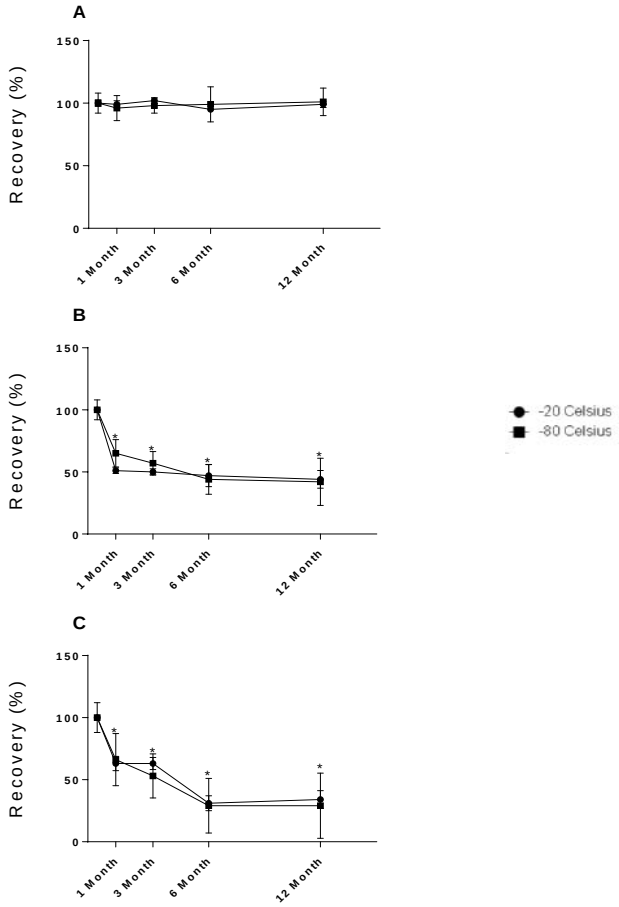
Study 1: Pre-Analytical Considerations when Measuring GLP-1 and Glucagon in Humans

Right now, more than 100 clinical studies (clinicaltrials.gov) include plasma levels of glucagon and GLP-1 as study outcomes. The accuracy of the methods used to assess plasma levels of glucagon and GLP-1 are of course crucial for correct interpretation of the data and the outcome(s).

Although it seems simple, adequate studies investigating the importance of pre-analytical sample handling (before the measurements) [24] have not been performed for glucagon and GLP-1 [25]. Before setting out in a larger study investigating the effects of short and long-term conditions on the stability of these two hormones, we first decided to clarify the importance of adding enzymes inhibitors to the tubes for analyte stability. For GLP-1, it is absolutely critical to add a DPP-4 inhibitor when estimating plasma levels of its active forms: 7-36NH_2 and 7-37 (because it is known that the N-terminus is particularly susceptible to cleavage by DPP-4). However, when evaluating the secretory profiles (the secretion), addition of a DPP-4 inhibitor is not crucial - at least when measurement is performed using a C-terminally specific assay (as the DPP-4 enzyme only cleaves the N-terminus of the GLP-1 molecule, which is not 'picked up' by a C-terminal antibody), but may not be the case when using sandwich ELISA as it is susceptible for endoproteolytic cleavage (such as the neutral endopeptidase 24.11) [26]. With regards to glucagon, studies from the 1980s showed some importance of adding trasylol (aprotinin), and this has been standard procedure ever since [27-29]. We (not formally part of the thesis) investigated the effect of trasylol on the stability of glucagons: By analyzing endogenous glucagon from human plasma samples taken with and without trasylol, we were able to demonstrate that adding trasylol to EDTA-coated tubes did not have impact on plasma concentrations of glucagon [30].

We then moved forward to investigate the importance of pre-analytical conditions, asking simple questions like 'For how long are glucagon and GLP-1 stable in human plasma?', 'what is the impact of freeze/thaw cycles?', 'is long-term storage safe?' and 'at which temperature should one keep the plasma samples?'

Although, laborious to do, the results turned out to be quite surprising and allowed us to provide the community with some general guidelines on 'how to take and store plasma samples for the later measurement of GLP-1 and glucagon' (**Study 1**). In short, both glucagon and GLP-1 are quite stable as long as they are kept on ice (actually up to 24 hours), but glucagon is extremely vulnerable to freezing (or the process of freezing and/or thawing), as illustrated by a 50% decrease in recoveries. In contrast, plasma concentrations of GLP-1 were only modestly affected of freeze/thaw cycles, as also reported by others [31]. Long-term storage of both does not seems to affect their stability and furthermore, the precise storage temperature (-20ºC versus -80ºC) seemed not to be important.



In panel A recoveries of intact GLP-1 are shown over a 12-month period. Panel B and C illustrate recoveries of glucagon estimated by a C-terminal (B) and an N-terminal RIA (C). Pooled plasma samples spiked with either GLP-1 or glucagon were stored at either -20ºC (circle) or -80ºC (square).

How can we explain such discrepancies in the stability of glucagon and GLP-1? One explanation may be that glucagon molecules have a tendency to fibrillate (formation of long insoluble protein structures, fibrils) [32, 33]. In other words, the loss of recovery is probably not due to enzymatic degradation but rather to physical chemistry related to the glucagon molecule. It has previously been shown that glucagon has an inherent risk of developing fibrils [34] (called twisting protofilaments), with both the formation and type of fibrillation depending on the concentration of glucagon (as studied using Fourier transform infrared spectroscopy). From an analytical perspective, generation of fibrils may result in falsely

lower levels of measurable glucagon (which is what we find). Cegla et al recently demonstrated, using two immune-based glucagon assays and mass-spectrometry, that addition of trasylol and/or a DPP-4 inhibitor does not influence the stability of glucagon [31, 35]. Furthermore, they also found that glucagon is susceptible to degradation, independent of measurement method. As we only studied the stability of glucagon in vitro, we cannot conclude anything about the stability of endogenous glucagon. It has been suggested that glucagon could be susceptible to activities of both DPP-4 [36] and NEP 24.11 [37]. With regards to DPP-4's potential role in glucagon degradation, the author of the thesis finds it rather unlikely that DPP-4 inhibition attenuates glucagon degradation as numbers of studies have shown lowered plasma glucagon levels in individuals treated with DPP-4 inhibitors [38] – this is probably due to the glucagonostatic effect of GLP-1 [39], which is increased due to increased levels of active GLP-1 prevented by the DPP-4 inhibitor [40] - and since others have reported glucagon as a poor DPP-4 substrate [41, 42]. On the other hand, as most of the above mentioned studies have applied C-terminally directed glucagon assays they would not be able to distinguish between intact glucagon and any potential DPP-4 truncated metabolite. The controversies of glucagon degradation and its dependency of DPP-4 activities merits further investigation.

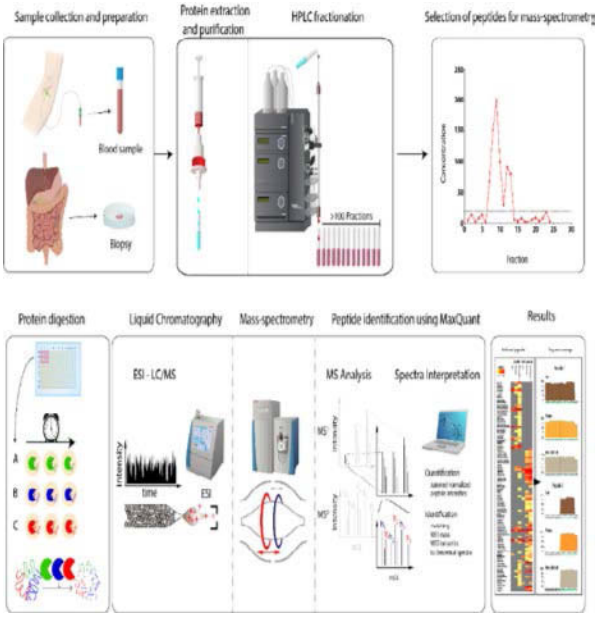
A better understanding of factors influencing the stability of glucagon could be important for the development of (more) stable glucagon isoform(s) [43] for subcutaneous delivery through 'dual-hormone pumps'[44] (close-loops pumps) for treatment of type 1 diabetes [45]. In addition, the findings obtained in **Study 1** highlight the importance for keeping in mind biochemistry-related concerns when designing, executing and analyzing human studies.

Study 2: An Unbiased Mass-Spectrometry Based Platform for Validation of ELISAs and Identification of Peptide Hormones

After studying the major pre-analytical caveats for glucagon and GLP-1 (**Study 1**), we turned towards accurate measurement of peptide hormones in human plasma with a focus on the proglucagon derived peptides: glucagon, OXM, and glicentin (**Study 2**). OXM was identified decades ago [46, 47] and found to have actions on both insulin secretion and gastric acid secretion, but also to act on both the hepatic glucagon receptor [48] as well as GLP-1 receptors. In agreement with these properties, it was also shown that this peptide may regulate appetite in humans [49, 50] (and therefore constitutes a potential drug target). However, only measurements based on a combination of subtraction assays and size exclusion chromatography had been available, and its potential implication in dysmetabolic conditions such as diabetes, obesity and gastric bypass surgery had not been adequately addressed. The entire glucagon amino acid sequence is part of the structure of both of the two gut-derived peptide hormones: OXM (consisting of glucagon + an 8 amino acid C-terminal elongation) and glicentin (which consists of the OXM sequence + a 32 amino acid N-terminal extension [47, 51]. We initially tried to use C-terminal antibodies (code name: 593 and 645) for the common C-terminus of the two molecules which, in principle, should detect OXM and glicentin equally, combined with differential elution/separation techniques, including solvent phase extraction (ethanol combined with various acids in different concentrations), solid phase extraction (C18 and C8 packed materials), and filters. Although we were able to reduce cross-reactivity to glicentin (from 70% to 20%), this approach was not sufficiently discriminatory for accurate measurement of OXM and glicentin in clinical plasma samples. A previous study by Laferrére et al [52] reported measurements of plasma levels of OXM (using a side-viewing antibody) during an OGTT before and one month after gastric bypass surgery in twenty obese women with type 2 diabetes. However, as we demonstrated in a separate study [5], the method used in that study is inaccurate and shows large cross-reactivity with other proglucagon related peptides (glicentin). The reported plasma levels seem overestimated by a factor of 10^3.

One way to solve this analytical problem is to detect the peptide of interest (in this case OXM) in an antibody-independent manner [53]. To that end, we developed an isolation and fractionation technique coupled to a highly sensitive mass-spectrometer **(Study 2)** (Orbitrap Elite and Qxactive from Thermo Fisher Scientific): Although this seems straightforward, it is nevertheless challenging. The first problem turned out to be that the high pressure liquid chromatography (HPLC) we used in Copenhagen did not have temperature control which resulted in 'season-depending' elution profile(s). Secondly, although the method may have a good specificity, sensitivity may be a challenge as the mass-spectrometry works in a 'data-dependent manner' allowing detection of only the 10 most abundant peptides (per timeslot). The latter is not the case in

time-of flight instruments (QTOF, currently being used in the Bruker instruments) and shown by others using an immune-based enrichment technique [54].



A mass-spectrometry based platform for detection of peptide hormones. Plasma are purified using C18 materials and subsequently proteins are separated using high-performance liquid chromatography. The samples are subjected to liquid chromatography coupled to mass spectrometry (LC–MS/MS) followed by data analysis using MaxQuant software suite.

In addition, some peptides may be what is termed 'bad flyers', making them poorly detectable by the mass-spectrometer; this for instance applies to glicentin (the N-terminally elongated form of OXM) which entails strict requirements for 1) negative and positive controls and 2) careful data analysis. Another limitation of the platform is that it is based on shotgun proteomics, which means that one does not identify the 'full length of the target protein' but rather multiple fragments (peptides) derived from enzymatic cleavages (typically induced by trypsin, chymotrypsin or Lys-C). Finally, less laborious methods are available, among others the SISCAPA technology (a commercial platform) [55], but in contrast to our method, these depend on existing antibody and/or calibrators (peptides of interest) and therefore do not allow detection and identification of new peptides or variants of existing ones. This limits their scientific applicability, whereas they may be very effective in clinical laboratory medicine. On the other hand, for the clinical application, why not just use a validated sandwich ELISA? In our case, we were interested in generating and applying a reliable OXM assay to be used for plasma samples from subjects with metabolic diseases and for that end, validation of the assay was of major importance whereas using a commercial platform for measuring a few plasma samples was not.

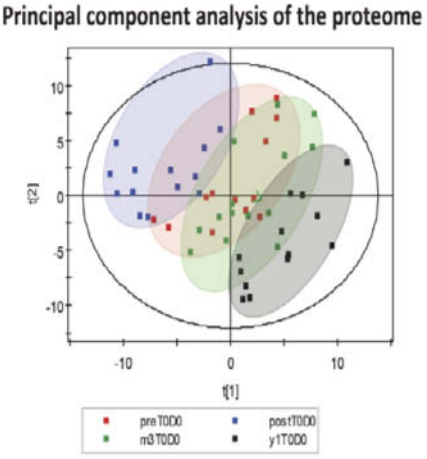
By using the mass spectrometry platform, we were able to confirm the accuracy of the ELISA (given that both methods identified identical 'OXM' HPLC peaks). In addition, this supports that OXM molecules are fully matured and, as such, are not (solely) a 'splice variant of glicentin' (although the latter may occur and is something we are looking into). Having validated a sandwich ELISA for OXM, we used it to show that OXM secretion during an OGTT is significantly lower in subjects with type 2 diabetes compared to healthy subjects, and is more than 10-fold increased after Roux-en-Y gastric bypass (RYGB) surgery. Finally, it seems that OXM is secreted from the gastrointestinal tract and in response to same stimuli as for GLP-1 (in line with the findings of OXM secretion from cultured GLP-1 cells [56]), suggesting that these peptides are co-distributed and co-secreted; this was of course expected, considering that they originate from same precursor, proglucagon [57]. The physiology of OXM has been investigated and described previously [58-62], and effects of treatment with chemically modified (stable) OXM agonists have been investigated [63, 64]. Collectively, OXM seems to have several effects on the gastrointestinal tract, including regulation of gastric motility and stimulating secretion of pancreatic juice (and perhaps, at pharmacological doses, insulin secretion [59, 65]). The OXM effects on food intake have awoken an interest in developing OXM for weight lowering therapy. Gastric bypass surgery has been associated with increased energy expenditure in rodents and in humans (although in humans, energy expenditure may actually fall, in parallel with the surgery-induced weight loss) [66-68], and it has been shown that OXM may increase energy expenditure in overweight humans [69], which seems to be consistent with the increased plasma levels of OXM we found after gastric bypass surgery (**Study 2**). In contrast, other studies have suggested that the change in energy expenditure after gastric bypass surgery fully reflects changes in body weight (thereby reducing resting energy expenditure) [68, 70, 71], while in a recent study in humans, there was no effect on energy expenditure in response to OXM infusion [72]. So in other words, the

pharmacology of OXM with respect to energy expenditure in humans is unclear at present, and investigations are hampered by lack of suitable assays – this is why the author believes it is crucial to develop, validate and apply a method like the one described in **Study 2**.

A somewhat surprising finding, given that valine pyrrolidide does not improve the stability of glucagon in pigs [42] or human plasma spiked with glucagon (**Study 1**), was the DPP-4 dependent degradation of OXM. Although we used valine pyrrolidide, which is not a fully selective DPP-4 inhibitor, the same effect has subsequently been observed using the same analytical methods, but in gastric bypass operated subjects treated with and without a selective DPP-4 inhibitor (sitagliptin) [73]. This, supported by previous studies [74, 75], indicates that OXM is a substrate for DPP-4 mediated degradation and, therefore, may be relevant when discussing the effects of DPP-4 inhibitors in clinical medicine. Nevertheless, DPP-4 inhibitors do not seem to induce a metabolically relevant weight loss [76, 77] (the inhibitors are typically classified as having weight neutral effects, although small, 1-2kg, reductions in body weight have been reported), but DPP-4 inhibitor induced increases in OXM levels may not be translated into changes in body weight due to the simultaneous inhibition of the formation of PYY 3-36 (known to inhibit appetite) [78]. In addition, neither of these studies [74, 75] investigated the plasma kinetics of OXM, but based the conclusions on *in-vitro* findings and observed differences in plasma levels before and after DPP-4 inhibition.

The intriguing question is whether OXM analogues can be developed for clinical use. As mentioned, a specific OXM receptor has not yet been identified; apparently OXM binds to both the GLP-1R and the glucagon receptor, albeit with 100-fold lower potency [50]. Therefore, there is no evidence on which to conclude whether, and to what extent, OXM contributes to improved glycemic control and reduced appetite in gastric bypass operated individuals, but our data do seem to support a role for OXM in diabetes (because of a reduced secretion) and after gastric bypass surgery. The absolute changes in plasma concentrations of OXM in patients with type 2 diabetes may not reach levels that would be expected to have a major impact of glycemic control, although the same argument has been made for GLP-1 (where only late phase postprandial secretion of GLP-1 was significantly lower during an OGTT in patients with type 2 diabetes compared to healthy controls [79]). Whether this decrease in OXM levels is of importance for the hyperglycemia of type 2 diabetes remains unclear (whereas the loss of the incretin effect certainly is [80]!).

Shifting back to the analytical improvement generated in **Study 2**, it deserves mentioning that the mass-spectrometry based platform developed here was also successfully used for the identification of glucagon in pancreatectomized subjects during an OGTT [81] and, as discussed later (**Study 4**), the same platform was also used to identify N-terminally elongated bioactive glucagon isoforms. With these examples, the applicability of this new method has been demonstrated, and it will of course be of great interest to use it for the potential identification of unknown or uncharacterized peptide hormones, for instance released after RYGB surgery. Together with a team at Novo Nordisk A/S and at Shanghai Biological Institute of Sciences (lead by Professor Jirai Wu), we are currently characterizing ~1000 proteins for which secretory responses are changed (negative and positive) after gastric bypass surgery. As illustrated below, using a principal component analysis (PCA), marked changes (seen as changes across time points: before (pre), 1 week after (post), 3 months after (m3) and 1 year after (y1)) may be reported on a global scale using mass-spectrometry based proteomics, which will facilitate identification of both known and unknown metabolic regulators.



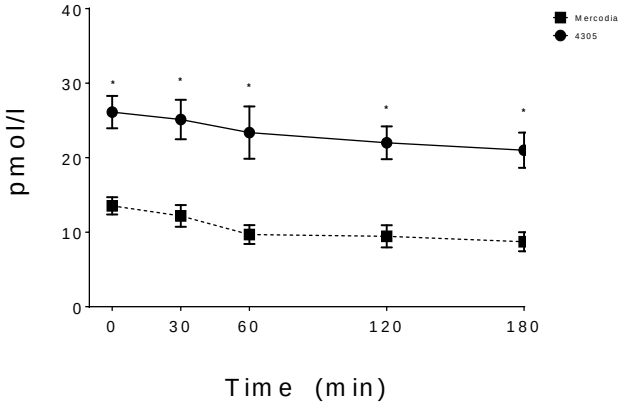
Two dimensional PCA analysis of changes in the plasma metabolome in 19 fasted obese subjects with and without type 2 diabetes before (red) and 1 week (blue), 3 month (green) and 12 months (black) after RYGB surgery. The figure illustrates the global changes of the plasma proteome induced by the RYGB surgery.

STUDY 3: MEASUREMENT OF GLUCAGON IN CLINICAL CONDITIONS

Measurement of glucagon is clinically important for diagnosis of glucagon-producing tumors [82, 83]. On the other hand, these tumors are relatively rare. It is, perhaps, of greater interest to measure plasma glucagon concentrations in rodents and humans during daily life and metabolic challenges [84]. Hypersecretion of glucagon may be important for the development of diabetes (the glucagonocentric hypothesis, first stipulated by Roger Unger [85, 86]) and to that end, several glucagon receptor antagonists have been developed [16, 87-89].

From the development of the first glucagon assay in 1959 [90], almost all research investigating hypersecretion of glucagon has used C-terminal glucagon assays (although this was not necessarily known at the time of development), whereas a few have used, what turned out to be unreliable, sandwich and or side-viewing glucagon assays [5]. Truly C-terminal assays may be highly specific and hence seemingly able to accurately measure plasma glucagon concentrations [91], but these are unable to discriminate between potential N-terminally elongated and/or truncated glucagon forms that may or may not occur in dysmetabolic conditions [92]. To what extent reported hypersecretion of glucagon actually represents 'true' pancreatic glucagon was not really known [93].

Mercodia (Uppsala, Sweden) developed, in collaboration with the author and Jens Juul Holst, a sandwich ELISA exclusively reacting with pancreatic glucagon (PG 33-61), therefore not recognizing OXM, glicentin, GLP-1 or N-terminally elongated or truncated forms of glucagon. We then measured and compared plasma responses of glucagon using the new sandwich ELISA specific for pancreatic glucagon to responses obtained using the 'traditional' C-terminal assay (code name 4305). Samples were obtained during OGTT or meal stimulations in clinical conditions with a described hypersecretion of glucagon, including subjects with type 2 diabetes, gastric bypass, vagotomy (for gastric disease) or kidney failure (end-stage renal disease). In all cases, except patients with kidney failure, (see figure below), secretory responses were similar (perhaps not identical - as in the case after gastric bypass surgery) whether measured with a C-terminal or the ELISA method. This suggests that the paradoxical hypersecretion of glucagon during an OGTT in subjects with type 2 diabetes does indeed represent a true biochemical hyperglucagonemia, whereas the hyperglucagonemia of subjects with kidney failure must be due to additional moieties with glucagon-like immunoreactivity [94].



*Plasma glucagon concentrations during an OGTT in patients with end-stage renal disease obtained with a C-terminal RIA (circle, 4305) and a new sandwich ELISA (square, Mercodia). * represents p<0.05 differences between the two methods.*

Neither may necessarily be novel observations, but importantly, they have now been reproduced using more specific analytical techniques (size-exclusion chromatography versus sandwich ELISA) (**Study 3**). In renal disease with reduced glomerular filtration rate (GFR), the plasma concentrations of several proteins may be increased. So one may ask whether the hyperglucagonemia or, more accurately, the high levels of glucagon-like immunoreactivity are simply due to reduced GFR [95]. I don't think there is a simple answer to that. If PG 1-61 is excreted from the kidneys, by receptor-mediated internalization (the glucagon receptor may be expressed in the thick ascending limb, distal tubule and the collecting duct [96]) or other unknown mechanism(s), it may aid our understanding of why subjects with kidney failure also show biochemical hyperglucagonemia (mainly due to increased levels of PG 1-61). Following this argument, plasma levels of glucagon (and perhaps GLP-1, given that the GLP-1 receptor has been identified in the afferent arteriole of the kidneys [97]) should also be elevated in subjects with kidney failure (or kidney disease), but this again seems to be complex, with the GLP-1 metabolite (GLP-1 9-36NH_2) but not intact GLP-1 (7-36NH_2) being increased when glomerular filtration is reduced [98]. Finally, one may speculate that some sort of link exists between the kidney and the pancreas (or the gut?) making the organ hypersecrete; however, we do not know of any evidence to support this.

STUDY 4: THE MOLECULAR HETEROGENEITY OF GLUCAGON IN HUMANS

The molecular heterogeneity of glucagon has primarily been investigated in subjects with glucagon producing tumors using RIA and size-exclusion chromatography [99, 100], but also normal subjects were investigated with this technique [101]. A study by Kuku et al showed that in subjects with chronic renal failure, a glucagon-like molecule (high molecular weight ~9000 Daltons) exists [102]. In addition, a similar high molecular weight component was observed in uremic pigs and identified by chromatography as PG 1-61 [103].

Inspired by the discrepancies, mentioned above and in **Study 3**, in plasma glucagon-like immunoreactivity as measured by the two different assay methods, we further analyzed plasma from kidney failure patients (same patients as in **Study 3**), but this time using the mass-spectrometry based platform developed in **Study 2**.

Using the mass-spectrometry based platform developed in **Study 2**, we analyzed pooled plasma from subjects with kidney failure (end-stage) and from healthy controls. We identified amino acid sequences identical with those of PG 1-61 with false discovery rate of less than 1% (meaning that the collective sequences detected combined have a less than 1% chance of being false positive). In order to provide further evidence, Mercodia (Uppsala, Sweden), in collaboration with this author and Jens Juul Holst, an ELISA specific for PG 1-61 and, in addition, subjected the same plasma samples to size-exclusion chromatography. Firstly, the summed plasma concentrations measured by sandwich ELISAs specific for PG 28-61 (glucagon) and for PG 1-61 resulted in comparable levels to that obtained by the C-terminal glucagon RIA. Secondly, size-exclusion chromatography demonstrated that indeed, two immunoreactive glucagon peaks could be identified using the C-terminal RIA, whereas the PG 1-61 ELISA reacted only with the one eluting first (Kd=0.3) and the PG 38-61 reacted only with the second later-eluting peak (Kd=0.8). Having established a method for detecting PG 1-61 and identified it in humans, we investigated its effects on glucose homeostasis using *in vitro*, *ex vivo* and *in vivo* models. In short, PG 1-61 binds the glucagon receptor causing a) insulin secretion and b) increased protein levels of several enzymes related to gluconeogenesis in the liver, which collectively seems to result in short-lasting (20min) increases in blood glucose. Blocking the glucagon receptor using siRNA guided knockdown in cultured beta-cells and cultured hepatocytes significantly attenuated the effects of PG 1-61, suggesting that the glucagon receptor is important for mediating its metabolic effects. Turning to the secretion of PG 1-61, our findings are somewhat unexpected, as one may have thought that changes in glucose levels would be the stimuli driving the hypersecretion of glucagon (with co-secretion of PG 1-61), but the picture was more complicated. Thus, in patients with anatomical gastrointestinal re-arrangements including Roux-En-Y gastric bypass surgery and total pancreatectomy, glucose but not protein was the major driver for PG 1-61 secretion, whereas for obese subjects and subjects with type 2 diabetes, protein and not glucose seemed to be the more prominent stimuli. How can we reconcile these results?

Let us first take a look at people with anatomical rearrangements. It has been speculated that after such operations, a reprogramming of intestinal L-cells is initiated that may result in glucose-induced glucagon secretion (in addition to glicentin and OXM) [104]. With regards to the patients after total pancreatectomy, we recently demonstrated, using the platform developed in **Study 2**, that during an oral but not intravenous glucose load, plasma levels of glucagon increase, showing that glucagon may be secreted from the gut in these patients. Several studies are aiming to delineate if and how changes in intestinal expressional profiles after gastric bypass surgery might explain the higher plasma levels of glucagon in these patients. A necessity for intestinally-derived glucagon appears to be co-expression of PC2 in proglucagon-producing cells. Using immunohistochemistry, Rhee et al were able to demonstrate such a co-existence after gastric bypass surgery [105]. However, in ongoing studies, it is being investigated whether fully matured glucagon can be extracted from intestinal biopsies obtained after post-RYGB surgery to support these results. This still does not explain the occurrence and secretion of PG 1-61 after oral glucose in these patients, but the findings are consistent with the appearance of molecular forms with the C-terminal (the '61' end) of PG 1-61 from the intestines. One could speculate that the intestinal proglucagon-expressing cells both express PC2 and PC1/3 (the latter is consistent with the increased secretion of several proglucagon derived peptide hormones including GLP-1, GLP-2, OXM and glicentin) and increase the transcription of proglucagon mRNA (preproglucagon) which, due to the expression of both PC1/3 and PC2, results in an L-cell-like splicing profile (GLP-1, GLP-2, OXM and

glicentin), an alpha-cell-like profile including glucagon, and finally a mixed-model, resulting in the generation of PG 1-61. Detection of PG 1-61 may, therefore, provide us with a 'snapshot' of what's going on in the enteroendocrine cells rather than, in itself, being a major regulator of glucose homeostasis.

Shifting to subjects with immunoreactive PG 1-61 in response to protein-rich meals, what seems clear is that both subjects with obesity and subjects with type 2 diabetes are capable of increasing plasma levels of PG 1-61 upon an intake of proteins but not of glucose alone. This is clearly in contrast to the gastric bypass operated and the pancreatectomized subjects. Proteins or amino acids are well-known stimuli for glucagon secretion (consider the classical arginine test employed for evaluating the secretory capacity for insulin and glucagon secretion in humans), whereas glucose is known to inhibit the secretion of glucagon (perhaps through closure of potassium ATP channels in pancreatic alpha-cells [106]). In subjects with type 2 diabetes, the hypersecretion of glucagon is called paradoxical since high glucose should inhibit its secretion. This has, for the last 5 decades or so, led to the glucagonocentric hypothesis which, in contrast to the bihormonal hypothesis (insulin deficiency and glucagon excess), suggests that it is the hypersecretion of glucagon per se that both contributes to the pathogenesis and the pathophysiology of diabetes. A potential weakness in the current rodent studies is that no proper model for type 2 diabetes exists. Thus, several studies have described an impact on glycemia, after inhibition of the glucagon signaling, in rodent models of type 1 diabetes (streptozotocin-induced, some included high fat diet) whereas other have not [107]. That said, glucagon receptor antagonists (being developed by several drug companies) do, in fact, seem to have glucose lowering capabilities in subjects with type 2 diabetes – which at least advocates for a central role of glucagon in the pathophysiology of type 2 diabetes, but nevertheless is not proof of its role in the pathogenesis of the disease. In our study (**Study 4**), the secretion of PG 1-61 in both obese subjects and subjects with type 2 diabetes could reflect a pancreas (alpha-cell) derived origin, which also is in line with the previous literature showing glucagon-like immunoreactivity with a larger molecular mass than native glucagon [108]. The question is then, how can it be that PG 1-61 comes from the alpha-cells and is NOT regulated by glucose, whereas glucagon is? Well, and as also discussed previously, there is increasing evidence for extra-pancreatic derived glucagon, be it from the gut or elsewhere, which does fit with these data. An important element is whether or not glucagon is actually formed in the gastrointestinal tract of subjects with type 2 diabetes and subjects after gastric bypass surgery. Studies regarding the latter are ongoing, but regarding the subjects with type 2 diabetes (T2DM), results of available studies are ambiguous. One way of answering this is to obtain tissue biopsies from the pancreas and the gastrointestinal tract from subjects with type 2 diabetes and subjects after gastric bypass surgery. Whereas the latter is feasible (in fact, we are doing this in collaboration with Prof. Filip K. Knop), the pancreas is of more challenge. Sadly, the studies described in PhD thesis do not allow conclusions to be drawn on from where PG 1-61 is generated and hence secreted, although we hope future studies may shed light on this exciting topic.

CONCLUSIONS AND PERSPECTIVES FOR FURTHER RESEARCH

The studies included in the PhD have described the development, validation and application of novel immune- and mass-spectrometry-based methods in order to accurately measure gastrointestinal hormones such as glucagon, OXM and GLP-1.

In **Study 1**, we systematically evaluated the preanalytical considerations that may be taken in order to adequately assess plasma levels of glucagon and GLP-1 in clinical studies. Of note, glucagon is particularly vulnerable (up to 50% variation in plasma levels), which emphasizes the notion that plasma samples from individual studies should be analyzed as one batch. The development of specific sandwich ELISAs for glucagon measurement may allow one to further dissect whether these variations in recoveries of glucagon are due to endoproteolytic cleavages or physical (fibrillation) related binding to the tubes wherein the plasma is obtained. What also would be interesting is to design a new study in which one obtained fasting plasma from healthy individuals and re-do (most) of the experiments in **Study 1,** since endogenous (by some, also termed native) glucagon may behave differently from exogenous glucagon as we used.

The methods developed (**Study 2**) and applied (**Study 3 and 4**) in this thesis constitute an important toolbox for current and future investigation on whether and how gut hormones are implicated in the pathogenesis and the pathophysiology of disease such as diabetes. In regards to technology, a variety of so-called multiplexing

instruments have been developed (e.g. Meso Scale Discovery, Luminex and Homogeneous Time Resolved Fluorescence), and although the current editions may lack sensitivity (most certainly due to the use of single-site antibodies), they provide a new and more holistic approach to screen plasma samples using a library of antibodies reacting with protein and peptide hormones which may be implicated in metabolism. Ongoing studies also include application of mass-spectrometry for large-scale identification of gut-related factors during weight loss, after gastric bypass surgery and in liver diseases (the latter is of great interest, as it seems that a parallel feedback system between the liver and the pancreas may exist in addition to that regulated by glucose). To identify low-abundant peptides in plasma, we generated an unbiased mass-spectrometry based platform that also allows validation of immune-based methods (**Study 2**). The application of such specific methods like mass-spectrometry-based proteomics may be a powerful resource to identify and verify known and unknown regulatory factors, as demonstrated here with PG 1-61 (**Study 4**) and finally to profile plasma from subjects with dysmetabolic conditions, as we recently have shown for obese subjects [109].

For the aspect of the OXM, some attention has been given to this hormone from a pharmacological perspective, as it inhibits appetite and may be a candidate for future treatment of obesity together with hormones like GLP-1 and PYY (potentially also glucagon) [14]. **In Study 2,** we demonstrated that secretory profiles of OXM are different in patients with type 2 diabetes and in gastric bypass operated individuals. It might have been interesting to further delineate the role of OXM in metabolic diseases such as diabetes, by which this new method (**Study 2**) allow us to do. Is the method for measuring OXM accurate? Well, it does cross-react with glicentin (<10%) but still it seems to be '*the worst form of OXM assays except for all those other assays that have been tried*'. Another limitation of the study (**Study 2**) is the missing cross-validation of both the ELISA and the mass-spectrometry based platform to other commercially available methods. We have, in fact, evaluated the current OXM assays in another publication (not included in the thesis) [5]. Regarding other mass-spectrometry based technologies for measurement of gut hormones; I do not see a productive way to use immune-based precipitation methods coupled to mass-spectrometry for measurement of low-abundant peptides such as OXM, due to varying recoveries (although this may be sufficient for pharmacological studies investigating the metabolism of the drug and so on).

The biology of glucagon remains an enigma [106] and, in addition, we can now add further complexity by demonstrating the molecular heterogeneity of glucagon in humans (**Study 3 and 4**). Whether such glucagon variants (PG 1-61) have any physiological impact, we do not know, but our findings do support that residual plasticity in enteroendocrine cells exists and may contribute to the remodeling of the secretory profile of proglucagon peptides. We need to consider that there are some limitations to both of these studies. Firstly, the ELISAs used in **Study 3** were prototype editions which may have affected the quality (the precision and accuracy) of the measurements in the clinical samples, and furthermore we should have measured plasma glucagon levels using the same tube for both the RIA and the new ELISA (this could have generated analytical bias of plasma concentrations shown in **Figure 3**). For the glucagon variant identified in **Study 4,** a major limitation (at least with respect to PG 1-61's role in pathophysiology) is the 100-fold lower potency compared to native glucagon combined with its relatively low concentrations identified in humans. These indicate that although PG 1-61 may be of general academic interest, it is unlikely of pharmacological interest, and furthermore its potential role in the increased hepatic glucose production observed in patients with type 2 diabetes is, at most, likely to be subtle.

In summary, accurate measurement of gut hormones is challenging and requires laboursome validation of the chosen immunoassays. The reliability of such methods is crucial for the understanding of the physiology of these hormones and, in particular, their implication in the pathophysiology of dysmetabolic diseases such as diabetes and obesity. However, new analytical methods such as mass-spectrometry based proteomics may improve the field of biochemical analysis through validation of current ELISAs and, more interestingly, identification and measurement of known and unknown regulatory factors, such as the glucagon variant identified and characterized within **Study 4**.

One may conclude that *the rise and fall of future studies do rely on the accuracy of the methods we choose.*

Summary

Towards the end of the 20th century, the number of subjects with diabetes and obesity rose exponentially. The discoveries of insulin-

and appetite-modulating chemical signals, including glucagon-like peptide-1 (GLP-1), secreted from the gastrointestinal system, led to development of a new group of drugs which now are being used for glucose-lowering therapy and weight loss. Understanding of the physiology of gut derived signals and their pathophysiological importance requires accurate measurements of their circulating levels. However, the assessment of these gut-derived hormones has been hampered by numerous preanalytical and analytical challenges. We focused on three members of the proglucagon family; glucagon, oxyntomodulin and GLP-1, aiming to meet both preanalytical and analytical challenges and to elucidate their implication in diseases including diabetes. First, we studied (**Study 1**) the preanalytical and storage conditions of GLP-1 and glucagon in humans, demonstrating that inappropriate sample handling may cause up to 50% variation in the results. Using robust measuring methods ensuring optimal conditions for preanalytical handling of these peptides, we then focused on plasma concentrations of glucagon and oxyntomodulin in different clinical conditions, including type 2 diabetes and bariatric surgery, because abnormal secretion of these hormones may represent early and specific signs of altered glucose metabolism. To that end, we developed an unbiased mass-spectrometry based platform for detection of low-abundant peptides, including the gut hormones (**Study 2**). Using the platform, we validated a new method for the measurement of oxyntomodulin, and in a series of *in vitro*, *ex vivo*, and clinical studies, we demonstrated that oxyntomodulin is co-distributed and co-secreted in response to glucose with GLP-1 and is degraded by dipeptidyl peptidase 4. Because oxyntomodulin has both GLP-1-like and glucagon-like bioactivity, the secretion of this hormone is of interest in both type 2 diabetes and bariatric surgery. Furthermore, using these newly developed methods, we subsequently were able to establish that elevated plasma concentrations of glucagon (hyperglucagonemia) in diseases (**Study 3**) may be due to either *a)* increased secretion of fully processed glucagon, as in subjects with diabetes or *b)* secretion of N-terminally elongated molecular forms (**Study 4**) in conditions including bariatric surgery and in diseases affecting the kidneys. This glucagon variant may be of importance for glucose homeostasis, as we were able to show that it, unexpectedly, activates the glucagon receptor, leading to increased glycogenolysis in hepatocytes and insulin secretion from pancreatic beta-cells. In summary, accurate measurements of gut-derived hormones are indeed crucial for understanding their biology in health and as well in disease. Mass-spectrometry based plasma proteomics is a powerful tool for the validation of these methods.

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